HELISOMA NEURONES IN THE CONSTRUCTION OF CIRCUITS IN VITRO

Scott R. Macfarlane

A Thesis Submitted for the Degree of MPhil
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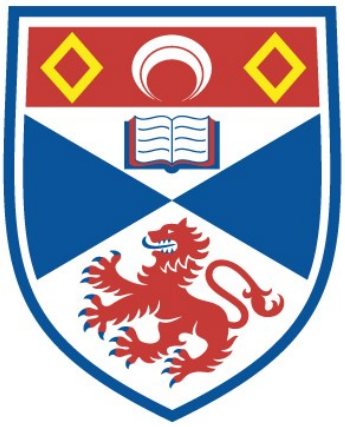

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Helisoma neurones in the construction of circuits *in vitro*

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DECLARATIONS

I, Scott R. Macfarlane, hereby certify that this thesis, which is approximately 26, 500 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Signed:

(Scott R. Macfarlane)

Date: 12/9/94.

I was admitted as a research student under Ordinance No. 12 in October 1991 and as a candidate for the degree of M. Phil. in June 1994; the higher study for which this is a record was carried out in the University of St. Andrews between 1991 and 1994.

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Date: 12/9/94.

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of M. Phil. in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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I acknowledge the support of the SERC in funding this research project.

DEDICATION

I dedicate this thesis to my family. Without their love and support I would never have completed this project.

ABBREVIATIONS

cAMP	adenosine 3′: 5′-cyclic monophosphate
GABA	gamma-aminobutyric acid
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid
KAce	potassium acetate
RNA	ribonucleic acid

UNITS

mm	millimetre
µm	micrometre
°C	degree Centigrade
l	litre
ml	millilitre
M	molar
mM	millimolar
mg	milligram
µg	microgram
kDa	kilo Dalton
MW	molecular weight
MΩ	mega Ohms
mV	millivolts
s	second

ABSTRACT

The work described here used the invertebrate neurone culture methodology to study neurones from the pond snail *Helisoma trivolvis*. Isolated neurones were used to form small circuits, the input/output properties of which were investigated electrophysiologically. The activity of the circuits were then described with respect to the intrinsic properties of the individual neurones and the synaptic connections between them.

Neurite extension from isolated neurones could be altered by making changes to the culture medium in which the neurones were maintained. Electrophysiological recordings made from pairs of neurones in culture revealed electrical, chemical and mixed connections. The purely chemical connections and the chemical component of the mixed connections were inhibitory in nature. Connections were not detected between all of the pairs. In many cases no connections at all were recorded between neurone pairs. Connections could also be obtained between neurone pairs if the two neurones were placed in culture on consecutive days.

Connections were still obtained when three neurones were placed together to form circuits, although such connections were found to be weak. The three neurone circuits formed were unable to produce any rhythmic output, due both to the weak synaptic connections present and intrinsic membrane properties of the neurones.

This study shows that invertebrate neurone culture is a viable way to study small circuits of neurones.

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CHAPTER 1
INTRODUCTION

The study of the nervous system has been a major preoccupation of scientists for many years. These studies have sought to explain the normal and abnormal function of nervous systems from the level of the whole organ down to subcellular events. In the course of these investigations anatomical, physiological, pharmacological and neurochemical techniques have all been used to help solve the problems encountered. A considerable amount of the interest in neurobiology has become directed at understanding of how nervous systems control the diverse behaviours expressed by animals.

Vertebrate nervous systems are highly organised and complex structures consisting of vast numbers of small neurones, with often diverse characteristics, interconnected by long thin axons and diffusely branched dendrites. These factors make the study of these nervous systems extremely difficult, particularly at a cellular level. This meant that a great deal of the earlier work on vertebrate preparations was of an anatomical nature, particularly those of Cajal towards the end of the nineteenth century, who first demonstrated that the brain consisted of individual cells (Cajal S. R., 1911). These anatomical studies have been important in the elucidation of nerve cell function. From this point much important work has been carried out on vertebrates using further anatomical and physiological methods to determine how neurones communicated with each other and with other body tissues. Studies of vertebrate nervous systems have ranged from whole animal behavioural and anatomical studies to brain slice and single neurone studies. The last of these techniques has proved extremely difficult in vertebrates. Even with the development of electrophysiological techniques, studying individual neurones from vertebrates remained difficult. This has meant that a great deal of work has been carried out using the extracellular recording technique to study populations of neurones. Even *in situ* studies, where the nervous system is left in the body of the animal, have proved difficult on vertebrate neurones. This has often been due to the instability of the preparation because of respiratory and cardiovascular activity in the animal making impalement of individual neurones, or brain areas difficult. Impaling neurones in such preparations has also proved difficult. This is because of the depth of tissue which the electrode often has to pass through before reaching its desired target.

Invertebrate animals offer experimenters comparatively 'simple' nervous systems consisting of far fewer neurones than those of their vertebrate counterparts. The nervous systems of invertebrates are generally organised into individual ganglia consisting of often large numbers of neurones. The neurones within each ganglion are in contact with neurones in other ganglia via connectives through which the neuronal axons pass. Neurones in invertebrate nervous systems are tend to be considerably larger and far easier to identify than those in vertebrate systems, due to their consistent position from one preparation to another. Individually identified invertebrate neurones have also been shown to possess characteristic morphology, connectivity and neurochemistry. Neurones from invertebrates are much more accessible than those from vertebrates, and they also tend to be physiologically tougher. A further advantage of using invertebrate systems for studying nervous system function is the relatively simple behavioural repertoire that these animals have. These behaviours are in many cases controlled by a relatively small number of neurones,making studies of such things as locomotion, feeding and respiration much simpler than in vertebrate systems.

Apart from the studies on invertebrate neurones which are involved in circuits, other aspects of the invertebrate nervous system have been exploited by investigators. In particular the squid giant axon has been used extensively in classical studies of membrane currents and action potential conduction (Hodgkin and Huxley, 1952). These studies in particular have established invertebrate preparations as important in nervous system studies.

The major moves to use invertebrates to study neuronal circuits were made in the 1960's (Wilson, 1961) and since then the field has expanded greatly, with more and more species being used. The major techniques used in these studies have been electrophysiological, recording from neurones in the ganglia which may or may not have been removed from the animal. Such studies have given us a great deal of information concerning neuronal circuits and their regulation.

In invertebrates single neurone studies have involved studying the cells whilst they are still in their respective ganglia and receiving inputs

from other neurones. These inputs could conceivably modulate the intrinsic properties of the individual nerve cell being studied, leading to possible misinterpretation of experimental results. This may be especially true if the study is of a circuit controlling a particular behaviour, where the neurones may be receiving sensory, or other information. In order to study single nerve cells in complete isolation many workers have used invertebrate neurones placed in cell culture conditions. This technique gives the experimenter the desired neurone in complete isolation from input from other body sources in an easily manipulated environment.

1.1 CELL CULTURE

Techniques for placing cells in culture conditions are now firmly established tools in the study of various vertebrate and invertebrate tissues. In particular those tissues from the kidney, liver and ovaries have been widely used in studies ranging from drug metabolism and toxicology, to the cellular mechanisms involved in cancer and other disease states. Placing cells into culture for study has the advantage of removing any inputs from other sources in the body, thereby allowing the tissue to be studied in isolation.

Cell cultures are essentially of two types; primary cell cultures and cultures of established cell lines. Primary cell cultures are formed from tissues which have been recently removed from the animal, and therefore provide a great deal of similarity to the *in vivo* situation. These cultures tend to have a limited life-span. Established cell cultures, or cell lines, consist of cells which have been genetically altered enabling them to survive indefinitely *in vitro*. The primary cell culture technique forms the focus of the present study.

Cell culture techniques were first used in the early part of the twentieth century. The earliest report of a tissue surviving after removal from the animal was made by Harrison (Harrison, 1907; Harrison, 1910). This work noted that pieces of frog embryo were capable of surviving and differentiating characteristically when isolated from the embryo and sealed in a chamber of lymph from the adult frog. Experiments designed to study the development of the nervous system allowed Harrison to use this technique to observe the growth of fibres into the lymph clot. From

these extensions he was able to observe varicosities and describe the neuronal growth cone. The work also reported that the isolated tissue could survive for one to four weeks.

Studies in the field of tissue culture expanded greatly from this point. These studies lead to improvements in the culture conditions to allow isolated tissue to survive for a longer period outside the animal. The problem of cell viability was mostly solved in experiments carried out by Carrel using embryonic chick tissue (Carrel, 1912). It was discovered that by replacing the old culture medium with fresh, the cells were capable of indefinite life *in vitro* . This work also demonstrated the cells were capable of growing and multiplying in suspension, and that by diluting the number of cells present, their growth could be increased. Another extremely important feature of this work was the observation that it was possible to keep a piece of heart tissue beating in culture for up to three months. This suggested that tissue cultivated *in vitro* could retain its normal function.

Early in the development of tissue culture techniques it was realised that the survival of tissue outside the body was dependant upon the nutrients available to the cells. After early experiments with Locke's solution (Lewis and Lewis, 1911), the number of artificial media available for cell survival and growth in culture has greatly increased. These artificial defined media which originally started as salines have become more complex with the addition of various nutrients. The addition of these nutrients allowed the lifespan and survival of cells in culture to be greatly improved. Basic defined media consist of a mixture of salts, amino acids, vitamins, hormones and energy sources. The discovery that adding substances such as cerebrospinal fluid or serum improved the survival and growth of cultured neurones lead to an upsurge in the use of conditioned media. These conditioned media are therefore defined media which have been supplemented by the addition of growth promoting factors. The added factor may be serum or haemolymph or, as in this study, an unknown brain-derived substance. Media to which such additions have been made can no longer be described as being defined, since the particular molecule or molecules responsible for the effect on the cells are not specifically identified.

The vast majority of cell culture work carried out since those early

experiments by Harrison has been on tissues other than that from the nervous system. Neurone culture was comparatively late in developing due to the difficulties encountered defining the media requirements for this tissue.

Vertebrate preparations are most widely used for studies of all tissue types in cell culture. Invertebrates have largely been ignored with regards to the use of culture techniques. The first neurone cultures from invertebrates began to appear in the 1970's, first with reports of neurones dissociated from the ganglia of the pond snail *Lymnaea stagnalis* (Kostenko *et al* , 1974), and then with reports of the isolation of neurones from the ganglia of the sea hare *Aplysia californica* (Kaczmarek *et al* , 1979) and the medicinal leech *Hirudo medicinalis* (Ready and Nicholls, 1979). These initial experiments set out the isolation techniques and culture conditions which are now widely used to study invertebrate neurones. In addition to the three species mentioned above, the pond snail *Helisoma trivolvis* has also received much attention in cell culture studies. The work on this animal has largely been based on previous *in situ* and *in vivo* experiments involving neurones from the buccal ganglia (Bulloch and Kater, 1981; Hadley *et al*, 1982, 1983; Haydon and Kater, 1988). These four invertebrates are the most popular ones for neurone culture studies, although the squid *Logligo paelei* and crustaceans are beginning to receive more attention in culture studies (Parsons and Chow, 1989; Cooke *et al*, 1989).

The majority of the invertebrate studies have involved the isolation of neurones from the nervous systems of adult animals. Embryonic neurones from invertebrates have also been successfully placed into culture conditions, where they were found to be capable of growing neurites (Goldberg *et al* , 1991; Beadle *et al*, 1989).

1.1.1 ENVIRONMENTAL FACTORS.

Many of the culture studies on invertebrates have looked at the effects that environmental elements have on neuronal development. Factors such as substrate, media and neurotransmitters have all been investigated in these experiments, the findings of which have proved important in the improvement of culture methodology and with relation to neural development *in vivo*.

In the field of culture methodology, several studies in *Aplysia, Helisoma* and leech have been important in establishing the media and substrate requirements for invertebrate neurones in cell culture. The use of animal-derived factors to aid in neurite extension has been reported in *Aplysia* (Schacher and Proshansky, 1983) and *Helisoma* (Wong *et al,* 1981 and 1983). In the study on *Aplysia* neurones it was shown that body fluids may be important on the *in vitro* development of neurites (Schacher and Proshansky, 1983). The report showed that if neurones were plated into medium containing haemolymph, both neuronal survival and neurite extension were increased. The neurites that were produced by the neurones were also seen to be affected by the addition of haemolymph to the culture medium. Increasing the haemolymph concentration of the medium led to a successive narrowing and thinning of the neurites produced by the neurones. In addition to this the branching ratio of the neurites was also increased. The results obtained may therefore suggest that a factor, or factors, present in the haemolymph may be important in the development of neurones *in vivo* and *in vitro.* This particular study was also important in establishing the importance of the initial axon segment of the neurone in controlling the formation of neurites.

Studies in *Helisoma* (Wong *et al,* 1981) and other invertebrates (Wong *et al,* 1983) established the importance of brain-derived factors in the development of neurones *in vitro.* The workers compared the appearance of neurones placed in defined medium alone to those co-cultured with intact central ganglia from the animal or plated in conditioned medium. Conditioned medium was obtained by incubating whole ganglia in basic defined medium. It was found that in defined medium neurones remained round, with little or no neurite extension apparent. However neurones in conditioned medium, or those in co-cultures with intact ganglia, showed a considerable amount of neurite growth. This increased neurite extension appeared to be due to the production of a ganglia-derived product, which could be removed by nitrocellulose filtration of the conditioned medium. This type of filtration would have removed proteins from the medium. In a further study these workers demonstrated that conditioning factors were involved in the regulation of neurite outgrowth in several species of gastropod mollusc (Wong *et al,* 1983). A more recent study into these

ganglia-derived factors, also using neurones from *Helisoma*, has shown that their release is dependant upon electrical activity within the central ganglia used in the production of the conditioned medium (Berdan and Ridgway, 1992). This study demonstrated that blocking electrical activity in incubating ganglia with the sodium channel blocker tetradotoxin, or the potassium channel blocker tetraethyl ammonium, removed the neurite promoting effect of the resultant conditioned medium.

A study on *Lymnaea stagnalis* neurones in cell culture suggested that the molecule responsible for the neurite extension in conditioned medium from gastropod mollusc species may be similar to nerve growth factor (Ridgway *et al*, 1990). This work showed that adding nerve growth factor to the culture medium lead to cell-specific neurite extension, with some neurones being induced to produce neurites while others were not.

Neuronal culture techniques have also been applied to leech neurones to investigate the effect of external environmental factors on neurite extension. The effect of divalent cations (Chiquet and Nicholls, 1987; Neely, 1993) and substrate (Chiquet and Nicholls, 1987; Grumbacher-Reinert, 1989; Ross *et al* , 1988; Neely, 1993) on the neurones have been studied most vigorously.

Studies into the role of divalent cations in neurite extension have reported that calcium, the ion of particular interest in these studies, may not be as important in neurite extension as may be imagined due to its role as an internal messenger. Calcium current measured from leech neurones in culture suggest that calcium uptake is less in the neuronal growth cone than in the neuronal cell body (Chiquet and Nicholls, 1987). The study does not state on which substrate the neurones were grown. This is important in light of a subsequent study which demonstrated that the substrate onto which the neurones are placed had consequences relating to the number of calcium channels on the neurites (Ross *et al*, 1988). In this report the calcium sensitive dye arsenazo III was used to image calcium ion concentration in the neurone and its neuritic branches. This gave the workers an indication of the density of calcium channels over the surface of the neurone. The experiments showed that neurones grown on concanavalin A substrate allowed less calcium entry in the processes than those grown on a substrate made from leech laminin extract. These latter findings are supported by two reports that

demonstrated a role for calcium in neurite retraction following depolarisation of a neurone (Grumbacher-Reinert and Nicholls, 1992; Neely, 1993). Neurite retraction was initiated by depolarising neurones using direct electrical stimulation or by incubating isolated leech neurones which were extending neurites in high potassium medium. This retraction was blocked by magnesium ions, indicating that the process may be calcium-dependant. These results were true for neurones developing on laminin substrate, but those on concanavalin A did not show any retraction in response to similar depolarisation. These reports therefore provide further evidence that substrate can alter calcium channel distribution on neurones in cell culture.

The type of substrate used can also have influences on the pattern of neurite extension and the synapses formed by isolated neurones (Chiquet and Nicholls, 1987; Grumbacher-Reinert, 1989; Lin and Levitan (1987). The first two of these studies demonstrated that leech neurones plated on concanavalin A and laminin substates produced different patterns of neurite extension on either side of the border between the substrates. On the concanavalin A substrate neurones produced thick curved neurites which branched often, whereas on the laminin based substrate neurites were long and thin with a lower frequency of branching. In isolated *Aplysia* neurones the use of concanavalin A as a substrate has an effect which discriminates against the formation of chemical connections (Lin and Levitan, 1987). This study found that electrical connections formed between neurones which normally formed chemical connections on other substrates.

Isolated *Helisoma* neurones have been extensively used to study the effects of the neurotransmitter serotonin on neurite extension (Haydon *et al* , 1984). In these experiments neurones B5 and B19 from the buccal ganglia and P5 (from the left pedal ganglion) were isolated and allowed to develop neurites. Serotonin was then applied and its effects on the neurites of each neurone type noted using time-lapse video recording. They found that neurite extension from the three neurones was effected by serotonin in different ways. Serotonin was found to inhibit neurite extension by neurone B19, whereas neurone B5 was not affected by the neurotransmitter. P5 was only transiently inhibited by the application of serotonin. The inhibition of neurite extension appeared to be due to an effect on the neuronal growth cone, and could be reversed by removing

the serotonin, suggesting that neurites extended in culture are capable of detecting neurotransmitters.

Work expanding on these experiments tested the effects of serotonin on synaptogenesis (Haydon *et al* , 1987). By inhibiting the extension of neurites from B19 with serotonin and allowing the B5 to continue with its extension to contact B19, the workers found that electrical connections never formed between the neurones. This was unlike the situation when both neurones were extending neurites simultaneously, when electrical connections were often detected. These results suggest that serotonin, or any substance which can differentially alter neurite extension, may provide important environmental controls during *in vivo* neural development and synaptogenesis. Further work on the effect of serotonin has shown an increase in calcium ion levels within the growth cones of the B19 neurone (Murrain *et al,* 1990). This effect was suggested to be via an effect on cAMP levels within the growth cone.

1.1.2 SYNAPTOGENESIS AND SYNAPTIC SPECIFICITY.

The major area of study using invertebrate neurones in cell culture has been synapse formation and specificity. *Aplysia, Helisoma* and leech neurones have all been used to investigate aspects of synaptogenesis. The neurone culture technique has great advantages for this type of study since the neurite extension from neurones can be controlled and interactions viewed. Most of the studies have relied upon neurones regenerating neurites in culture, allowing the two neurones to contact each other. However in many cases the participating neurones can be placed in contact via their somata or axon segments the ensure some area of contact exists. Both the neurones of interest can also easily be recorded from in cell culture conditions.

Electrical coupling between bag cells from *Aplysia* was first reported in the late 1970's (Kaczmarek *et al,* 1979). This showed that neurones in cell culture were capable of forming connections that were present in the intact animal. In the early 1980's it was shown that leech neurones were also able to become synaptically connected in cell culture (Fuchs *et al,* 1981). These leech neurones were seen to form both appropriate and inappropriate electrical connections. In a few cases Retzius cells were noted as forming novel inhibitory chemical connections with each other,

where electrical coupling would have been expected to mirror the *in vivo* situation. These early studies demonstrated the ability of isolated neurones to form not only connections that would usually occur in the animal, but also to form connections that would not usually be seen.

The area of the neuronal membrane which participates in the formation of synapses has been well reported. Experiments were carried out where leech neurones were manipulated so that different parts of the neurones were in contact (Nicholls *et al,* 1990). The workers found that chemical connections were detected if the two axon stumps were placed in contact, or if the axon stump of one neurone was placed in contact with the soma of the second neurone. These connections became electrical after 24 hours. If contact was made via the somata or neurites, electrical connections were initially found. These synapses became chemical after 3-8 days in the case of soma-soma contacts, and after 24 hours in the case of neurite contacts. The workers concluded that contact via axon stumps or axon stumps and somata were the most reliable way of obtaining chemical synapse formation between two neurones. Axon-soma contacts involving leech neurones were further used to study calcium currents during synapse formation (Fernandez-De Miguel, 1992). This study reported that the calcium current in the prejunctional axon stump and postjunctional soma were increased in size after synapse formation, whilst those in the prejunctional soma and postjunctional axon stump were reduced. This therefore gives some evidence for a redistribution of calcium channels on both synaptic partners during synapse formation.

The majority of studies on synapse formation have been carried out using neurones which are in contact via mutually extending neurites. One noteable exception however is a study which used neurones B5 and B19 from the buccal ganglia of *Helisoma* (Haydon, 1988). These two neurones, which do not form a connection with each other *in vivo,* were shown to be capable of forming a cholinergic chemical connection from B5 to B19 in cell culture, if their somata were placed in direct contact. The culture conditions into which these neurones were placed was unfavourable to neurite extension. This technique gave the experimenters a unique opportunity to record directly from the pre- and postsynaptic sites, allowing them to study synaptogenesis at both areas. In further studies using these neurones, where they were allowed to

extend neurites, the ability of the two neurones to release neurotransmitter was investigated (Haydon and Zoran, 1989; Zoran *et al*, 1990). These studies demonstrated that neurone B5 was able to release acetylcholine after as little as three seconds contact with a neurone, whereas B19 required prolonged contact with its appropriate muscle target before it could release any transmitter. The release of neurotransmitters from different neurones would therefore appear to be determined by different signals.

The ability of neurones to form synapses differentially has been reported in *Aplysia* (Schacher, 1985). This study demonstrated that the metacerebral cell, from the cerebral ganglia, would only form chemical connections via neurites from the appropriate branch of the axon with its targets B1 and B2. If these neurones were placed next to the branch of the metacerebral cell which innervates the posterior lip nerve, no connection was seen. This shows that the two branches of the neuronal axon can detect the presence of the appropriate target. Evidence was also presented which suggested that target neurones can have an effect on neurite extension from the axons of the neurone which innervates them.

The remaining studies into synaptogenesis in culture have concentrated on the specificity of the connections formed. These studies have been extensively carried out using neurones from leech and *Aplysia.* In general, the work has established that neurones in cell culture form a set of stereotyped connections with neurones which it may or may not connect with *in vivo.* This means that one neurone may form one set of connections *in vivo,* but when placed in culture it may be capable of forming synapses with additional neurones with which it normally has no contact. It is also possible that two neurones which are synaptically connected *in vivo* may form a different synaptic connection in culture.

In *Aplysia* the *in vitro* connectivity of two different neurones has been reported. Interneurone L10 from the abdominal ganglion was initially reported as forming connections in culture only with neurones which it synapses with *in vivo* (Camardo *et al,* 1983). The L10 neurone was shown to connect with its *in vivo* targets, the upper left quadrant neurones, in cell culture whilst avoiding making synaptic connections with neurones from the right upper quadrant, which it has no contact

with *in vivo*. L10 was therefore reported as having the same specificity, with regards to the left and right upper quadrant neurones, since synapse formation was the same *in vivo* and in cell culture. A second study using neurone L10 has altered this view of L10 specificity (Kleinfeld *et al*, 1990(a)). This report demonstrated that L10 was indeed able to connect with some of its *in vivo* partners in cell culture, however some of the connections were of a different sign. For example, the L10 synapses onto neurones R15 and R16 via excitatory connections and onto neurone L7 via a dual action synapse. In culture the connections from L10 to R15 and R16 were reported as being inhibitory, as was that from L10 to L7. Another alteration to the L10 connectivity was seen in the synapses between L10 and L7, L11, L12 and L13. These connections were reported as being unidirectional from L10 *in vivo*, whereas in culture these pairs were shown to form bidirectional chemical synapses. It was also found that L10 neurones in cell culture were able to receive an input from neurone L14A, with which L10 has no contact at all *in vivo*. This difference in synaptic specificity has also been reported for neurone R2 from *Aplysia*, which was shown to form a different set of connections in cell culture to that which it forms *in vivo* (Schacher *et al*, 1985).

The work carried out on synaptic specificity in *Aplysia* has been widely supported by many studies using neurones from the leech. These reports, as with those on *Aplysia* neurones, have found that neurones in cell culture form specific sets of connections which are not always identical to those seen *in vivo* (Arechiga *et al*, 1986; Vyklicky and Nicholls, 1987; Nicholls *et al*, 1990). The results from leech and *Aplysia* on synaptic specificity show that in cell culture the specific interactions between neurones that lead to the formation of synapses *in vivo* are replaced by a less stringent set of interactions.

A study of synapse formation using neurones from *Aplysia* has shown that the amount of contact between neurones has an effect on synapse formation (Hawver and Schacher, 1992). This report demonstrated that chemical connections could be detected between L10 interneurones and right upper quadrant neurones if contact between the pair was forced by intertwining their axon segments. The workers concluded that without forced contact between these pairs there is limited contact between neurones as neurites from L10 would avoid contacting neurites from novel targets. In pairs where contact was forced, the area of

overlap between the axons was high, and the extending neurites also showed more overlap, leading to an increased chance of synapse formation.

Another aspect of synapse formation studied using neurones in cell culture has been the segregation of sensory inputs over the surface of the target neurone (Bank and Schacher, 1992). This study used L7 motorneurones and two sensory neurones from *Aplysia*, and showed that initial contact by the sensory neurones onto L7 is random and unsegregated. However as time in culture increased the sensory inputs, visualised in the study as varicosities on the axon of L7, became spatially segregated over the initial axon segment of L7. This segregation led to inputs from one sensory neurone being arranged over one area of the axon whilst inputs from the second sensory neurone became situated on a different area of the axon. If the two sensory neurones were connected to each other by an electrical synapse the segregation of the varicosities from the two neurones was disrupted, and the inputs were reported to be intermingled to a greater degree. This observed breakdown in the segregation may be due to the increased communication between the two sensory neurones via the gap junctions of the electrical connection. The two electrically connected neurones may act as one neurone due to this increase in cellular communication.

1.1.3 GROWTH CONES.

Placing neurones in cell culture gives investigators a unique opportunity to study areas of the neurone which are not normally very accessible. Possibly the best example of such an area of a neurone is the growth cone. This part of a neurone is usually found in the developing nervous systems of embryos where visualising them is difficult, as is visualising growth cones in the neuropil of regenerating adult nervous systems. Neuronal cell culture techniques provide investigators with easier access to growth cones than *in situ* studies.

Some of the studies into growth cones in culture have previously been discussed, particularly with relation to the effect of serotonin and substrate. Other studies have been made into the effect of internal calcium on growth cone behaviour (Rehder and Kater, 1992) and the ability of growth cones to synthesise protein (Davis *et al*, 1992).

The filopodia which extend from the leading edge of the growth cone have been suggested as being involved in probing the environment for the appropriate stimuli for further growth of the axon or neurite. This means that control of the filopodia has important consequences for the development of the neurone. By depolarising neurones in culture using high potassium media, or by applying a calcium ionophore, it was reported that filopodia extended from *Helisoma* neuronal growth cones could be shown to transiently increase in length and eventually be retracted (Rehder and Kater, 1992). These findings strongly implicate calcium ions in the control of filopodial extension, and therefore suggest a role for calcium ions in the growth cone as a means of controlling neurite extension.

Protein synthesis in isolated *Aplysia* neurones has previously been reported (Ambron *et al,* 1985). This report demonstrated that neurones extending neurites in cell culture were able to synthesise various proteins and transport them into their neurites. A more recent study in *Helisoma* has shown that growth cones which have been isolated from the rest of the neurone are capable of synthesising protein (Davis *et al,* 1992). These experiments demonstrated that isolated growth cones could take up radioactively labelled leucine and incorporate it into proteins. This suggests that neuronal growth cones contain the machinery necessary for the synthesis of proteins, independent of input from the neuronal cell body. Information obtained from ultrastructural studies supports this biochemical evidence, showing that the isolated growth cones contain polyribosomes, organelles required for the production of proteins (Davis *et al,* 1992).

1.1.4 BIOCHEMISTRY AND ULTRASTRUCTURE

Invertebrate neurone culture has been used to study neuronal ultrastructure and biochemistry. As described above, protein synthesis in isolated neurones from *Aplysia* (Ambron *et al,* 1985) and isolated growth cones from *Helisoma* (Davis *et al,* 1992) have been reported. The earliest report of invertebrate neurone culture also suggests that the biochemistry of isolated neurones is maintained, since neurones from *Lymnaea* were reported as being capable of synthesising RNA in cell culture (Kostenko *et al,* 1974). This observation was supported by a later

study using isolated neurones from *Lymnaea* and *Helix*, both of which were shown to incorporate radioactive uridine in the medium into their RNA (Bocharova *et al*, 1975).

Two of the studies which noted biochemical aspects of invertebrate neurones in cell culture also looked at the ultrastructure of the neurones (Bocharova *et al*, 1975; Davis *et al*, 1992). The findings of these reports suggested that the ultrastructure of isolated neurones does not differ greatly from that observed in the whole ganglia, with all the organelles and fine structure being preserved. Scanning electron microscopy on the membrane of isolated neurones revealed that the membrane displayed a high degree of folding, and that small sections of glial cells and neuronal processes were still attached to the isolated neurone. When these areas of contact between the neuronal processes and isolated soma were studied ultrastructurally, the workers found the areas to be structurally similar to synapses.

Leech neurones have also been used in ultrastructural studies in cell culture (Fuchs *et al*, 1981; Nicholls *et al*, 1990). The first of the reports supports the previous findings in *Lymnaea* and *Helix* which suggested that neuronal ultrastructure is preserved in the isolated neurones (Fuchs *et al*, 1981). The second of the electron microscopy studies of leech neurones investigated synapses formed between neurones in cell culture (Nicholls *et al*, 1990). The workers concluded that the ultrastructure of the synapses formed between Retzius and P sensory neurones and between two Retzius neurones show the principal characteristics of chemical synapses *in situ*. For example, dense-core vesicles and synaptic thickening were reported on the presynaptic sides of these junctions.

1.2 NEURONAL CIRCUITS.

The nervous systems of both vertebrates and invertebrates are organised into networks of interconnected neurones which control various aspects of the animals behavioural repertoire. These circuits can consist of either large or small numbers of neurones. One thing that has become increasingly clear is that, no matter how large or small circuit is, the complexity of output is not solely the result of the synaptic connections between the participating neurones. The output of a circuit is reliant on the synaptic connectivity, synaptic properties and intrinsic

membrane properties of the component neurones.

Understanding the principles behind the operation of neuronal networks is obviously of great importance to our understanding of the nervous systems functions. Studies of neuronal circuits in vertebrate animals have been somewhat restricted, due to the complexity and organisation of the nervous systems in these species. However studies on lower vertebrates and their embryos, such as the lamprey and *Xenopus laevis* tadpoles, have allowed many investigators to study principles involved in vertebrate locomotion (Grillner *et al*, 1992; Arshavsky *et al* , 1993). A great many studies have been carried out on invertebrate species, in the hope that principles learned in these 'simpler' nervous systems may hold true for the vertebrates.

The first report of the elucidation of a neuronal circuit came in the early 1970's, after investigation of the escape response in crayfish (Zucker *et al* , 1971). From this point on the number of invertebrate circuits studied has greatly expanded. The circuits which tend to be chosen are those which underlie rhythmic behaviours such as feeding, digestion, locomotion, respiration and heartbeat. This rhythmicity gives experimenters a repeated pattern of activity making studies easier to carry out.

Most commonly studied are the interactions of neurones in central pattern generator networks. These arrays of neurones control the output of many behavioural networks in invertebrates, and the principles learned from them are being widely applied to vertebrate circuits.

1.2.1 THE STOMATOGASTRIC GANGLION.

Since the 1970's the crustacean stomatogastric ganglion has been the subject of a great deal of intensive study. Several different crustaceans have been used to study this part of the crustacean nervous system, which is responsible for the control of muscles in the stomach. The ganglion contains thirty neurones which are divided into circuits controlling different parts of the stomach; the pylorus, the gastric mill and the cardiac sac. The neurones controlling the cardiac sac rhythm are not wholly set in the stomatogastric ganglion, but are spread between several ganglia within the nervous system.

1.2.1.1 **The Pyloric Network.**

The circuit controlling the pyloric rhythm is a subset of fourteen stomatogastric neurones, which are probably the most vigorously studied in this preparation. Electrical and inhibitory chemical connections are present within the circuit, with reciprocal chemical inhibition playing an important role in the operation of the network. In a comprehensive study of this circuit the connections between neurones, the membrane properties of the neurones and the overall network properties of the pyloric circuit were studied (Miller and Selverston, 1982a and b; Eisen and Marder, 1982). These studies looked to explain the generated pattern obtained from the network. Of particular interest was the appearance and origin of oscillation observed in the system, and how the connections between the neurones lead to the timing of neuronal firing within the pattern. Techniques such as dye-inactivation, sucrose nerve block and pharmacological dissection were used to study individual neurones or small parts of the circuit. The investigators concluded that the oscillation observed during network activity was induced by external inputs, except for those seen in the pacemaker AB neurone which was suggested to be an endogenous burster. Other neurones in the network are able to produce oscillations when under the influence of inputs from other parts of the nervous system. The importance of cellular properties of individual neurones, and also the properties of the synaptic interactions between neurones, have also been studied in relation to pattern generation in the stomatogastric ganglion (Hartline *et al,* 1988).

Further studies on this network have focussed on the modulatory inputs that it receives (Hooper and Marder, 1987; Nusbaum *et al,* 1992). These studies have demonstrated that neurotransmitters, particularly monoamines and neuropeptides, are capable of inducing different patterns of activity from the same network. Transmitters which can alter the activity of the AB neurone have been shown to have profound effects on the network output (Harris-Warrick and Flamm, 1987). This subtle modulation of the circuit by different neurotransmitters can lead to a situation where one 'hard-wired' circuit may have several modes of activity. These variants may possibly relate to the different behavioural states of the animal (Harris-Warrick *et al,* 1989).

Although these neurotransmitters do not modulate all of the

neurones in the network, the uneffected neurones still have important roles in the modulated circuitry via their connections with effected neurones. This observation is made since modulatory substances are not merely required to change the activity of a few neurones in a network, but the overall activity of the network. This must include both neurones sensitive to the action of the modulator, and those insensitive to its action.

Many of the modulatory substances that have been shown to effect the neurones of the pyloric network have been identified in input neurones to the ganglion from other areas of the crustacean nervous system.

1.2.1.2 **The Gastric Mill Network.**

The eleven neurones of the gastric mill network, ten motorneurones and one interneurone, are all found in the stomatogastric ganglion. These neurones form a central pattern generator that controls the three teeth in the stomach. This circuit has not been as extensively studied as the pyloric network, but the circuit interactions and modulation of the circuit have been investigated (Heinzel, 1988a and b; Heinzel and Selverston, 1988).

The circuit has been shown to function in two different functional modes, squeeze mode and grind mode (Heinzel, 1988a). In addition to this there have been a few reports of the neurones of the gastric mill network and the pyloric network merging to form new functional circuits (Weimann *et al* , 1991; Meyrand *et al* , 1994). These findings suggest that the number of possible patterns of output from a network may allow small networks to fuse and control different behaviours under the influence of different neurotransmitters.

More recently neurones from the stomatogastric nervous system have been studied in cell culture (Panchin *et al,* 1993; Turrigiano and Marder, 1993).

1.2.2 *TRITONIA* ESCAPE SWIMMING

The circuit which controls the escape swimming behaviour in the marine mollusc *Tritonia diomedia* is another invertebrate network which has been studied in some detail. Initial work on *Tritonia*

characterised the swimming behaviour, its stimuli and the neurones involved (Willows, 1967; Willows and Hoyle, 1969). The identification of the neurones making up the network was completed when two groups of cerebral ganglia interneurones were discovered (Getting, 1977) and a role for the I interneurone was also suggested. Since this study the escape swimming network has received intensive study (Getting *et al,* 1980; Lennard *et al,* 1980; Getting, 1981; Hume and Getting, 1982a and b; Getting, 1983a and b; Getting and Dekin, 1985).

The network controlling the escape behaviour consists of fourteen neurones, seven in each cerebral ganglion. Six of these seven neurones are postulated to form the circuit responsible for the swimming behaviour. These are C2, the dorsal swim interneurone group and the ventral swim interneurone group. The I interneurone is suggested to be the neurone which gates the circuit and that this interneurone is active during normal behaviour to ensure that the escape swimming behaviour is not expressed.

The wiring diagram of this circuit shows that the dorsal swim interneurones are mutually excitatory. However depolarising one of these neurones inhibits the others, due to polysynaptic inhibition of the dorsal neurones by the I interneurone. This polysynaptic pathway is dominant during normal behaviour, thus preventing swimming from occuring. When the appropriate sensory inputs are stimulated, the C2 neurone becomes active and strongly inhibits the I neurone, thus allowing mutual excitation of the dorsal swim interneurones. Under these conditions the escape behaviour can take place. Only when the C2 neurone becomes less active than the I neurone does the non-swimming mode take over (Getting and Dekin, 1985). This circuit therefore provides an excellent example of the observed synaptic connections being unable to predict the final output of the circuit.

Studies on the roles played by neurotransmitters in modulating this circuit have not been reported. Since the synaptic potentials evoked between members of this circuit have been shown to be subtle, and in many cases multiphasic (Getting, 1981), neuromodulators may be found to have profound effects on the circuit output. These effects may not be confined to altering cellular properties, but effects on the synaptic properties of these neurones may also be important.

1.2.3 *LYMNAEA* FEEDING

Neuronal circuits controlling feeding behaviour have been a popular area of study. In particular the feeding system of the pond snail *Lymnaea stagnalis*. This system was first studied in detail in the late 1970's (Benjamin *et al*, 1979; Benjamin and Rose, 1979; Rose and Benjamin, 1979), and the circuit has been extensively investigated since these initial experiments.

The neurones which participate in the network are found in each of the paired buccal ganglia. Although ten classes of motorneurones and three types of modulatory neurones are involved in the function of the feeding system, the patterned output of the circuit is produced by three kinds of interneurone; N1, N2 and N3. These groups of interneurones which make up the central pattern generator are capable of producing the feeding rhythm without input from other neurones. The three classes of interneurone are connected by purely chemical connections. However the output of the circuit cannot be explained by the synaptic connections alone. Intrinsic membrane properties such as postinhibitory rebound (N3), postburst hyperpolarisation (N2) and endogenous bursting (N1) play an important role in shaping the emergent output of the pattern generator.

Closer investigation of the central pattern generator found that the electrically coupled N1 neurones were capable of reverberation, allowing them to produce an oscillation to drive the feeding rhythm (Rose and Benjamin, 1981a and b). These further investigations of the network allowed the study of neurones which have inputs onto the pattern generating network (Elliot and Benjamin, 1985a and b). The slow oscillator neurone was of particular interest, and was subsequently shown to provide the main drive for the production of the feeding rhythm. This neurone was found to be modulatory, since it is not required for the production of the rhythm, but is necessary to drive the rhythm at the frequency seen *in vivo*. Neurones have also been discovered in the cerebral ganglia which have modulatory effects on the central pattern generator (McCrohan and Benjamin, 1980; McCrohan, 1984a and b; McCrohan *et al*, 1989).

The modulatory effects of various neurotransmitters have been tested on the feeding pattern (Tuersley and McCrohan, 1988; McCrohan *et al*, 1989). These studies show that the differential effects of such neurotransmitters as serotonin and FMRF-amide could form the basis of modulatory control of the feeding pattern in *Lymnaea*.

The central pattern generator network controlling feeding in *Lymnaea* is an example of a circuit whose output has been directly linked to the phases of the muscle behaviour it controls. This makes the feeding central pattern generator of this animal an excellent system to study rhythm generation and its modulation.

1.2.4 *LYMNAEA* RESPIRATION.

The control of vertebrate breathing is difficult to study at a neuronal level, and invertebrates have therefore become popular tools in the study of respiratory behaviour. Although several invertebrate studies have been carried out, those which used the pulmonate pond snail *Lymnaea* are of particular interest, since this central pattern generator has been reconstructed in neuronal cell culture.

The central pattern generator for this behaviour is small, consisting of three interneurones, and again demonstrates that the 'hard-wired' synaptic connections between neurones are not all that is important in pattern generation (Syed *et al* , 1991; Syed and Winlow, 1991). As in other networks discussed, intrinsic membrane properties play an important role in the production of the final output of this network. Reciprocal inhibitory connections between neurones also play an important part in this circuit.

By placing the three neurones into cell culture the workers were able to study the interactions between them more closely. The effect that intrinsic properties of the individual neurones have on the emergent pattern of activity could also be studied in this system (Syed *et al* , 1990). The method was particularly strengthened by the observation that the neurones were capable of producing patterned output identical to that produced *in vivo*. This manipulation of a small central pattern generator has opened up a wide range of possible investigations into the mechanisms underlying pattern generation, where external influences

have been removed. Using the culture system has particular applications to studying the role played by intrinsic membrane properties in the production of circuit output.

1.2.5 *HELISOMA* FEEDING.

The feeding system in *Helisoma* is one which has been studied for many years and the basis for the feeding pattern has previously been described in detail (Kater, 1974). The premotor neurones which innervate the motorneurones and provide the feeding pattern are known as cyberchron neurones. The motorneurones are divided into protractor and retractor types. Electrical coupling is present between the members of the protractor group and also between the retractor group, and aids simultaneous firing within populations.

This feeding pattern consists of alternate bursts of activity in the protractor and retractor neurones. The drive and timing of these bursts are controlled by the cyberchron network, which excites the retractor motorneurones causing them to fire. During this period the cyberchron neurones inhibit the protractor neurones, which fire due to their ability for postinhibitory rebound. This is another example of the role played by intrinsic membrane properties in pattern generation. Sensory input to the protractor neurones from mechanoreceptors has an important role in terminating the bursts in these neurones (Kater and Rowell, 1973).

Further studies of the cyberchron network have demonstrated that the output of this circuit is likely to be due to electrical interactions between the neurones of the network itself, making it unlikely that one of the cyberchron neurones acts as an endogenously bursting neurone which drives the rest of the network (Kanenko *et al* , 1978; Merickel and Gray, 1980).

Studies of the modulation of the feeding pattern of *Helisoma* have not been carried out as vigorously as in the stomatogastric system or *Lymnaea* feeding system. The work that has been reported suggests that the monoamines serotonin and dopamine are important in the control of the feeding pattern (Granzow and Kater, 1977; Trimble and Barker, 1984). Neurones containing these transmitters have been shown to be

present in the system, and may therefore be providing intrinsic control of the feeding motor program (Granzow and Kater, 1977; Trimble *et al* , 1984). The serotonergic input from the C1 neurones of the cerebral ganglia appears analogous to the cerebral giant cell input to the *Lymnaea* feeding system (McCrohan and Benjamin, 1980). This observation seems to point towards a common feature in the control of these two feeding patterns. In addition to the role played by the monoamine transmitters, peptide transmitters have also been shown to affect the feeding pattern in *Helisoma* (Murphy *et al,* 1985). The peptides FMRF-amide and small cardioactive peptide B were shown to inhibit and initiate the feeding pattern, respectively. Furthermore the report demonstrated that immunoreactivity to these peptides was present in the buccal ganglia of *Helisoma.* This further supports the possiblity that these peptides may have a role in the control of the motor pattern for feeding.

More recently the amino acid transmitter GABA has been implicated in the control of the feeding rhythm in *Helisoma* (Richmond *et al,* 1994). This work showed that GABA can initiate cyclical feeding output by a picrotoxin insensitive mechanism. In addition to this GABA was also shown to have an inhibitory effect on the buccal motorneurone B19 via a picotoxin sensitive mechanism. This neurotransmitter therefore has two distinct actions which are capable of modulating feeding activity in this snail. The report suggests that these effects are mediated via molluscan forms of $GABA_A$ and $GABA_B$ receptors. A role for this neurotransmitter in the control is supported by the discovery of GABAergic neurones in the buccal ganglia (Richmond *et al,* 1991).

The control of feeding in *Helisoma* appears markedly different from that in *Lymnaea,* but the two systems do appear to share a similar mechanism for activating their feeding patterns. In *Helisoma* the output of the electrically coupled cyberchron network appears to be generated by reverberation of the output of pacemaker cyberchrons throughout the network. This leads to strong activation of the network, allowing it to drive the feeding pattern. The N1 network from the feeding system of *Lymnaea* would appear to operate along similar lines, with reverberation amongst this electrically coupled network aiding in the production of feeding activity. The major difference between the two systems lies with the phasing of input from the central pattern generating networks to the motorneurones. In *Helisoma* the simple alternation of

bursts seen in the two classes of motorneurones are produced by the different synaptic connections from the cyberchron network. These are excitatory in the case of the retractor neurones are inhibitory in the case of the protractor neurones. The motorneurones receive only one phase of input during the feeding pattern. The motorneurones in *Lymnaea* receive three phases of input however, corresponding to the inputs from the three classes of interneurone which make up the central pattern generator.

Covering the vast number of invertebrate circuits which have been studied over the years would be an extremely difficult task. Work in other species such as leech and *Clione* (Satterlie, 1985) have also been of great importance in the elucidation of many of the properties of pattern generating networks. More recently the focus of study has moved onto how circuit output can be shaped by neurotransmitters and sensory inputs. Of particular interest has been the roles played by monoamines and neuropeptides.

Many of the principles derived from the study of invertebrates have been found to hold true for vertebrate neuronal circuits. As has been found with invertebrate networks the emergent output of vertebrate circuits requires not only neuronal connectivity, but also intrinsic membrane properties of individual neurones. Investigators have also noted similarities in the organisation of vertebrate and invertebrate circuits (Arshansky *et al* , 1993; Pearson, 1993).

1.2.6 CIRCUIT RECONSTRUCTION IN CELL CULTURE.

Invertebrate neurones in cell culture have been used to investigate the function of neuronal circuits. These studies have exploited the ability to isolate the component neurones of a circuit to study the network without inputs from other neurones or sensory receptors (for review see Bulloch and Syed, 1992).

The first report of an isolated neuronal circuit being studied in cell culture appeared in the mid-1980's when neurones comprising the circuit involved in the gill withdrawal reflex in *Aplysia* were used (Rayport and Schacher, 1986). Motorneurone L7 was placed in culture along with two LE sensory neurones to make up the basic circuit. A modulatory

neurone, the serotonergic metacerebral cell, was also added in some cases. It was noted that the neurones of the circuit behaved as previously reported *in situ,* showing homosynaptic depression of the sensory-motorneurone synapses and heterosynaptic facilitation of the same synapses by the modulatory input from the metacerebral cell. This report provides an example of the use of cell culture technique to study neuronal circuits where the technique gives access to the synapses involved, allowing more detailed studies of their modulation.

Further studies of this circuit have looked at the facilitation which is induced by some neurotransmitters at the sensory-motorneurone synapse (Schacher *et al,* 1990) and also at the facilitation which is induced by actvity in the presynaptic neurone when it is paired with neurotransmitter action (Eliot *et al,* 1994).

One of the most notable reports of the reconstruction of a neuronal circuit *in vitro* saw the neurones controlling the respiratory rhythm in the pond snail *Lymnaea* isolated and recorded from in cell culture (Syed *et al* , 1990). Using the techniques developed in *Aplysia* and *Helisoma* for isolating identified individual neurones, this group were able to place the three neurones from this circuit, VD4, LPeD1 and IP3, into culture conditions. The neurones were found to extend neurites and reform the appropriate connections allowing the breathing rhythm to be accurately reproduced. This lead to the conclusion that the three neurones were sufficient to account for the rhythm, and that placing neurones in culture conditions was a viable way to study the activity of neuronal circuitry.

Neurones from *Aplysia* have also been used for the construction of small circuits which have been shown to be capable of producing stable patterns of output (Kleinfeld *et al,* 1990(b)). The neurones in these circuits were shown to interact with each other in accordance with a predicted model, and the output of the circuits could be shaped by altering the currents injected into the neurones. One of the circuits was constructed using neurone L10 and left upper quadrant neurones, which were interconnected by reciprocal inhibitory connections, and the other was formed using L7 and L12 neurones which were reciprocally excitatory. Both circuits were found to be capable of existing in two stable output states. In the case of the inhibitory circuit, one stable state was achieved when L10 was firing and the left upper quadrant neurone was

quiescient, and the second was seen when the left upper quadrant neurone was firing and the L10 neurone was silent. The excitatory circuit showed two stable states, one when both neurones were silent and one when both neurones were firing. The workers postulate that the control of these circuits by injected current may be analogous to the control of circuits *in vivo* by inputs from modulatory neurones. These simple circuits were also postulated to act in a similar manner to groups of neurones, for example the reciprocally inhibitory circuit may be likend to that proposed for groups of antagonistic motorneurones in the half-centre model (Brown, 1914).

One further circuit from the pond snail *Helisoma* has also been reconstructed in cell culture. Neurones RPeD1, LPeD1 from the pedal ganglia and VD4 from the visceral ganglion of the central ganglionic ring have been successfully shown to form appropriate chemical connections present *in vivo* (Syed *et al* , 1993). These synapses were recorded between neurones which were extending neurites. The overall activity of this circuit was not assessed however, and only the individual connections were studied.

1.3 COMPUTER MODELS.

An increasingly important technique in the study of neuronal networks is the ability to use computer models to simulate the circuit of interest (Selverston, 1993). This technique has been widely applied to invertebrate preparations, particularly leech (Calabrese and De Schutter, 1992). Applications have also been discovered in vertebrate preparations such as the lamprey (Grillner *et al* , 1991). The uses of this technique are still developing, but will undoubtedly provide invaluable support to investigators in the future, as an extra tool to back up their experimentation on real biological systems.

1.4 THE PROJECT.

The work described here proposes to use the culture techniques available for *Helisoma* to construct a small circuit of neurones. The neurones used in the study have previously been reported as surviving in cell culture, and have also been shown to form chemical synapses. Once in culture the circuits will be electrophysiologically investigated

using the intracellular current clamp technique. The emergent patterns of activity recorded from the circuits will be related to the synaptic connectivity and intrinsic properties of the individual neurones involved.

CHAPTER 2
METHODS

2.1 ANIMALS.

Adult specimens of the freshwater snail *Helisoma trivolvis* , with a shell diameter of 10-20 mm, were used in all the experiments described. These animals were easily bred and maintained in the laboratory, being fed daily on fish food or lettuce. The tanks containing the animals were aerated and maintained at a constant temperature of 23 - 24°C.

2.2 REMOVAL OF GANGLIA.

2.2.1 Central Ganglia.

The dissection of ganglia from the animals was carried out under sterile conditions. The preparations were visualised using a binocular dissecting microscope.

The body of the animal was first removed from the shell by cutting around the middle of the shell from the head to the tail, as shown in figure 1(a), using a pair of small dissecting scissors. This allowed the shell to be pulled apart to give two halves. The animal was still attached to one of the halves via the aductor muscle. The snail was then teased from the shell using a pair of forceps, and placed for thirty minutes into antibiotic saline (composition of the saline is described under Solutions on page 32) containing 20% "Listerine" to sterilise them. Animals were then transfered to a "Sylgard" coated dissecting dish, where they were pinned out in normal antibiotic saline as shown in figure 1(b). An incision was made with iridectomy scissors from the base of the head to the mouth, passing along an assumed midline. The muscular tissue of the buccal mass was then pulled forward of the animal and pinned. The central and buccal ganglia were in clear view at this point.

The oesophagus was then cut at a point posterior to the central ganglia. By pulling the oesophagus forward towards the buccal mass the central ganglia were left free for removal. The ganglia were carefully gripped between the cerebral ganglia with a pair of sharpened No. 5 forceps, and the whole organ was lifted slightly. All of the nerves to the periphery of the animal were then easily cut with iridectomy scissors. The organ was then placed into defined medium (composition of defined medium is described under Solutions on page 33) until required for further dissection or preparation of conditioned medium.

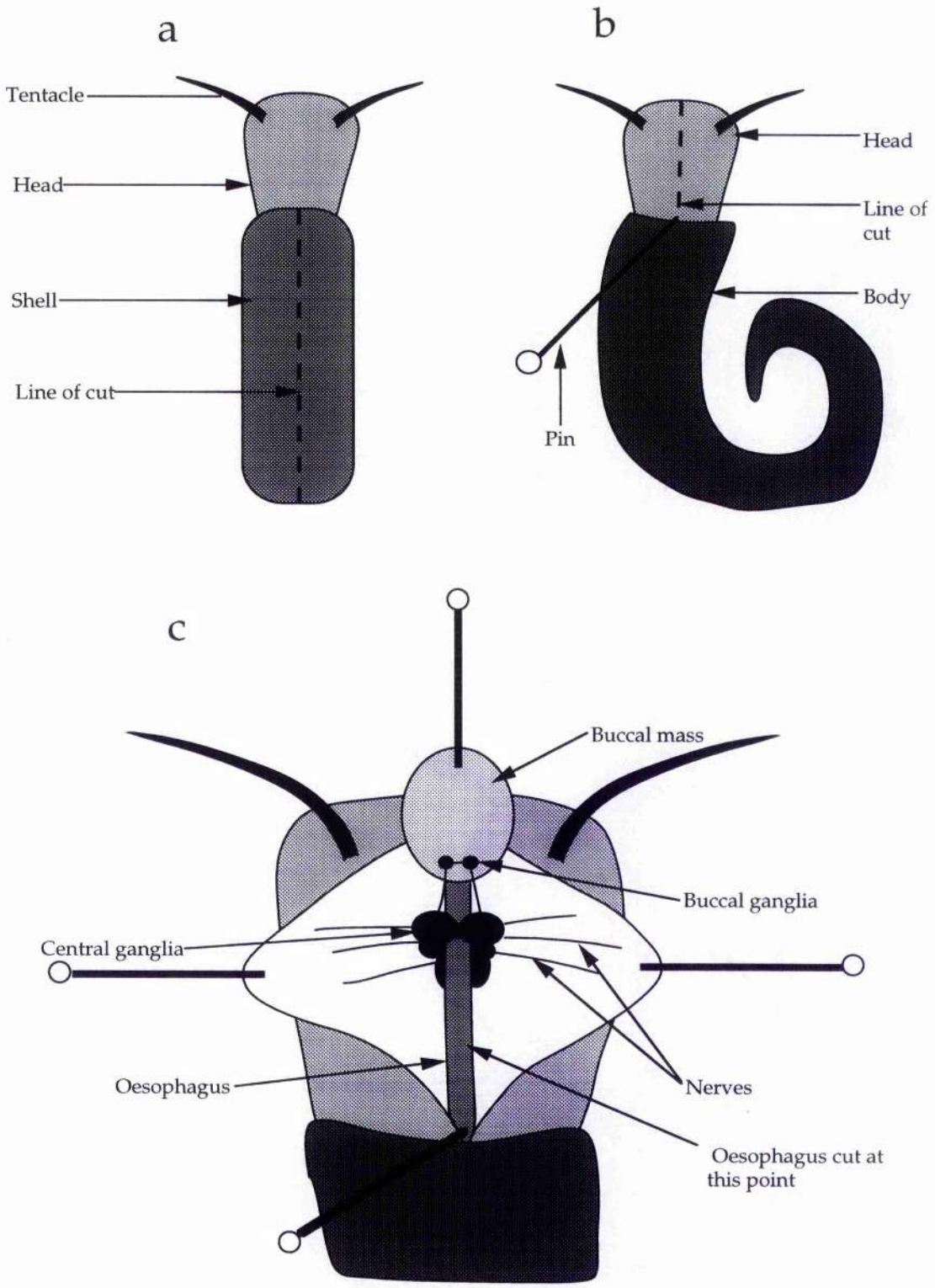
Figure 1.

Diagram showing the stages of the dissection to remove the ganglia from the snail.

(a) Deshelling. This shows the line along which the shell was cut to allow the animal to be removed.

(b) This diagram shows how the animal was pinned for for the initial incision. The cut was made from just above the collar of the animal, where the pin was positioned, to the front of the head.

(c) This represents the animal after the cut has been made. At this stage the buccal mass has been pulled forward of the animal and pinned down, leaving the buccal and central ganglia of the animal in clear view. The oesophagus of the snail projects from the buccal mass through the central ganglia to the body of the animal. This organ must be cut, at the point shown, to free the central ganglia for removal.


a
Tentacle
Head
Shell
Line of cut
b
Head
Line of cut
Body
Pin
c
Buccal mass
Buccal ganglia
Central ganglia
Oesophagus
Nerves
Oesophagus cut at this point

2.2.2 Buccal Ganglia.

The buccal ganglia were removed using the following method. Firstly the oesophagus was cut free of the buccal mass, leaving it attached to the buccal ganglia via the oesophageal nerve trunks. The piece of oesophagus was then pulled carefully away from the animal using a pair of forceps, to allow the nerves from the ganglia to the buccal mass to be observed. A small piece of the buccal musculature adjacent to the posteriobuccal nerves was cut from the main buccal mass, freeing the ganglia from the animal. Removal of a small section of the oesophagus and buccal muscle with the buccal ganglia was necessary to aid with pinning the ganglia at a later stage. The preparations were then placed into defined medium until required for further dissection.

2.3 FINE DISSECTION.

2.3.1 Central Ganglia.

The ganglia were incubated in 0.2% trypsin (Sigma, type III) in defined medium for 25 minutes. This allowed enzymatic digestion of the sheath of connective tissue surrounding the neurones of the ganglia. The ganglia were removed, washed in fresh defined medium for 10 minutes and then placed in a "Sylgard" coated Cel-Cult 35mm culture dish containing 2ml of defined medium.

The thick connective between the two cerebral ganglia was cut using iridectomy scissors. Both cerebral ganglia were then pulled flat out on the "Sylgard" surface and pinned with fine entomological pins. Preparations were set up to show the dorsal surface of the ganglia, as shown in figure 2. Another pin was then placed through the anal and parietal nerves, emanating from the visceral and parietal ganglia respectively.

Excess layers of connective tissue, previously softened by the enzymatic treatment, were removed using sharpened No. 5 dissecting forceps. The neurones were now in clear view through the final thin layer of the connective sheath. Neurones to be isolated were identified by means of position in the ganglia, size and pigmentation. Both the visceral and parietal ganglia were removed at this point, using fine

Figure 2.

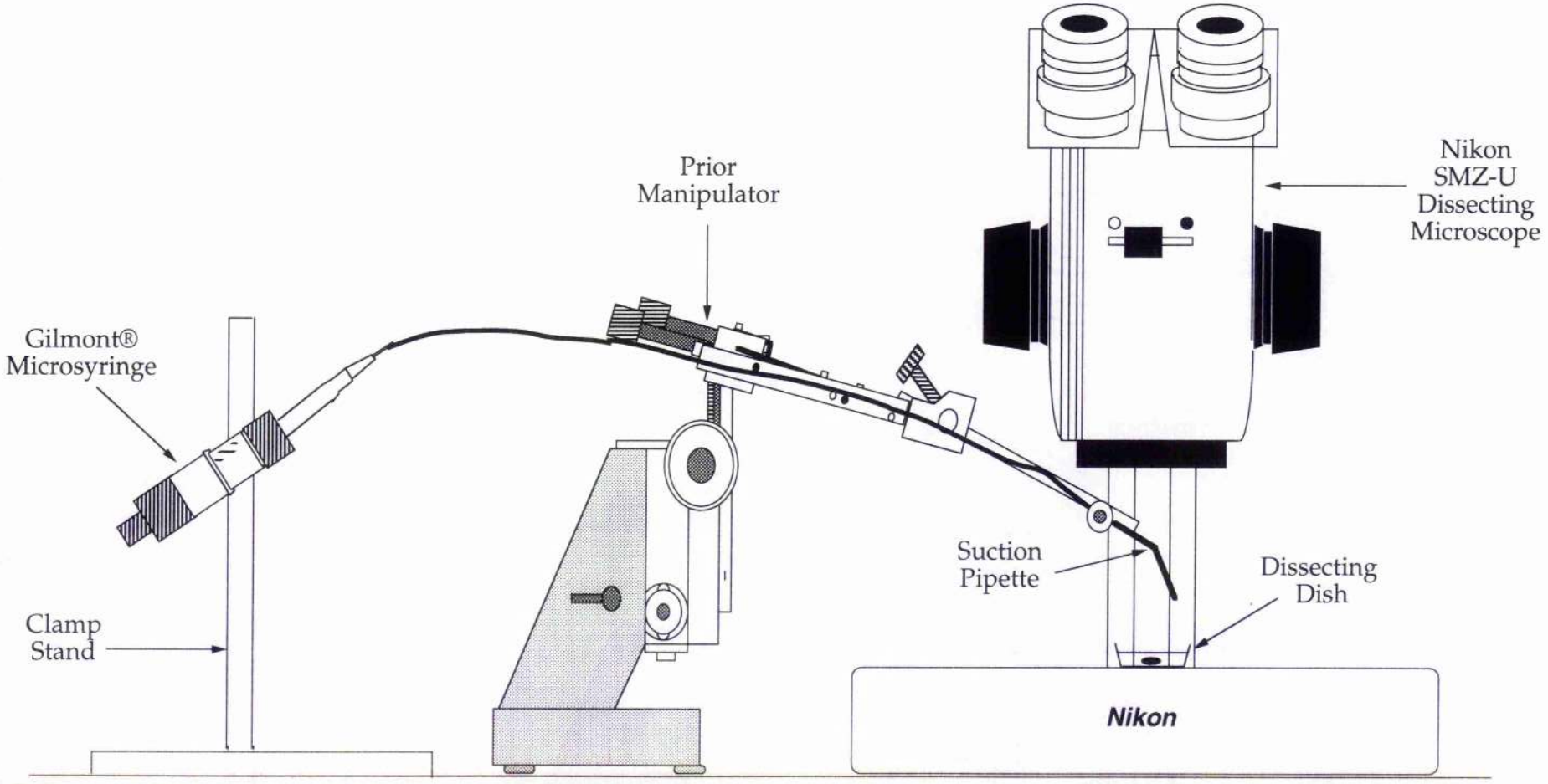
Upper plate. Photograph showing the central ganglia of *Helisoma* after it has been pinned for dissection. Scale bar= 200 ≡m.

Lower plate. Photograph of the buccal ganglia after being pinned for dissection. The pieces of buccal mass (top) and oesophagus (bottom) were removed with the ganglia to aid with pinning the preparation. Scale bar= 1mm.


200µm

Figure 3.

Schematic diagram of the apparatus used during the isolation of neurones into culture.


Prior
Manipulator
Nikon
SMZ-U
Dissecting
Microscope
Gilmont®
Microsyringe
Suction
Pipette
Dissecting
Dish
Clamp
Stand
Nikon

iridectomy scissors. This was done to allow the suction pipette easier access to the pedal ganglia and also because severing the axons of the giant dopamine neurone (GDN) and large serotonin neurone (LSN) made them easier to remove under suction. Additional pins were then placed through the pedal nerves to rotate the pedal ganglia making the GDN and LSN easier to see, and also to hold the preparation more firmly in place.

A small slit was made in the ganglionic sheath using an electrolytically sharpened tungsten microknife in order to expose the neurones for removal. Gentle pressure was then applied to the ganglion using sharpened No. 5 dissecting forceps to cause the desired cell to 'pop-out' of the ganglion. The neurones were then removed to a fire-polished suction pipette, tip diameter 100µm, by the application of gentle suction from a Gilmont® microsyringe. A Prior micro-manipulator was used to control the position of the suction pipette. The cell body and its attached axon was gradually pulled from the ganglion until the axon could be severed mechanically or was severed due to the applied suction. Neurones were then transferred from the suction pipette into a poly-L-lysine (Sigma) coated culture dish (either Cel-Cult 35 mm dishes or Falcon 3001) containing 2 ml of medium. The type of medium used varied (see Results). Isolated neurones were allowed to adhere to the substrate for 15 minutes before any further cells were added to the dish. Neurones were left at room temperature, 20-23°C, for 15 to 18 hours before being placed into a humidified B and T Unitemp incubator at a temperature of 22°C.

The apparatus used in neurone isolation is shown in figure 3.

2.3.2 Buccal Ganglia.

The buccal ganglia preparations were incubated in 0.2% trypsin (Sigma, type III) solution in defined medium for 20 minutes. Ganglia were washed in fresh defined medium for 10 minutes before being transferred to a "Sylgard" coated Cel-Cult 35mm culture dish containing a further 2ml of defined medium. The buccal ganglia were pinned for dissection by placing entomological pins through the small pieces of buccal muscle and oesophagus removed along with the ganglia. Other pins were then placed through the remnants of the cerebrobuccal connectives,

heterobuccal and ventrobuccal nerves. Figure 2 shows the buccal ganglia after being pinned as described.

Unlike the central ganglia, the buccal ganglia did not have any excess connective tissue surrounding them, and the neurones could be immediately observed through the ganglionic sheath. It was possible to identify specific neurones because of their size, pigmentation and position in the ganglia.

2.4 PREPARATION OF CONDITIONED MEDIUM.

Ganglia to be used in the preparation of conditioned medium were first washed in 5 ml of defined medium for 30 minutes. The ganglia were placed, at two ganglia per ml, into Cell Cult culture dishes containing a suitable amount of defined medium.

The dishes were then placed into a humidified B and T Unitemp incubator at 22°C for 72 hours. Ganglia were removed from the medium and placed into 5 ml of fresh defined medium for 30 minutes. The medium in which the ganglia had been incubated was then filtered through a 0.2 μm HT-200 Tuffryn® membrane filter (Gelman Sciences) into plastic centrifuge tubes (Cel-Cult). This filtration removed any solid material and bacterial or fungal contamination that may have been present. The centrifuge tubes were then labelled and frozen at -22°C for future use. Conditioned medium stored in this way was used within two weeks of initial freezing.

Once the used ganglia had been washed for 30 minutes, they were used to prepare a second batch of conditioned medium. After the second use, the ganglia were discarded.

2.5 PREPARATION OF POLY-L-LYSINE COATED CULTURE DISHES.

This procedure was carried out in a Gelaire laminar flow hood (Gelman Instruments), under sterile conditions.

25 ml of 0.15 M Tris buffer was passed through a 0.2 μm Millipore nitrocellulose filter into 25 mg of poly-L-lysine hydrobromide (Sigma No. 7890, MW 25,000 or No. 6516, MW 10,000) to give a poly-L-lysine solution of 1 mg/ml. 1ml of this solution was then pipetted into 35 mm Cell-Cult

or Falcon 3001 plastic culture dishes, using an autoclaved glass Pasteur pipette. The lids were then returned to the dishes and they were left in the flow cabinet for 24 hours.

Excess poly-L-lysine was removed from the culture dishes under suction using an autoclaved glass Pasteur, bent in a Bunsen burner flame near one end. The dishes were then washed twice with sterile milli Q water, which was again removed under suction, before being washed with sterile *Helisoma* antibiotic saline (ABS) for 30 minutes. This saline was then removed and the plates were washed a further two times with sterile milli Q water. The dishes were then left to dry in the flow hood for 2 hours before being placed in a plastic tray, sealed and left for 48 hours before use.

2.6 STERILE PROCEDURES.

All dissecting instruments were cleaned before and after use with a solution of 70% alcohol in distilled water, as were all surfaces used during the dissection. The dissection itself was carried out in a Perspex hood to prevent dust and other airbourne particles falling into any of the solutions used. The suction pipette apparatus was rinsed thoroughly with 70% alcohol after use, as were all the "Sylgard" coated dissecting dishes.

The work surface in the fume hood was wiped down with a gauze swab which had been soaked in 70% alcohol solution before and after any work. All bottles placed in the fume hood were also swabbed, as well as being flamed thoroughly on opening both before and after use.

2.7 PHOTOGRAPHY

The photographs of the buccal and central ganglia were taken using a Contax RTS camera mounted on a Zeiss Technival 2 microscope.

Photographs of the cultured neurones were taken using a Contax RTS camera containing black and white AGFAPAN 32 film. For all culture photography the camera was mounted upon a Zeiss IM 35 inverted microscope.

2.8 ELECTROPHYSIOLOGY

Electrodes for intracellular recordings were made from filamented borosilicate glass capillaries with an external diameter of 1.5 mm (Clark Electromedical Instruments). These capillaries were cut to the required size and placed on a simple Narshige horizontal electrode puller where they were pulled to the desired tip size. The electrode resistances were typically 10-30 MΩ when filled with 1M KCl or 1M KAce.

Intracellular recordings were made from the isolated neuronal somata after one to four days in the culture conditions. Neurones were visualised on a Zeiss IM 35 or Olympus CK binocular inverted microscope. Both instruments had built in light sources.

Electrodes were mounted on two Leitz micromanipulators. When recordings were made from three cells, a Narshige micromanipulator was added to the set-up.

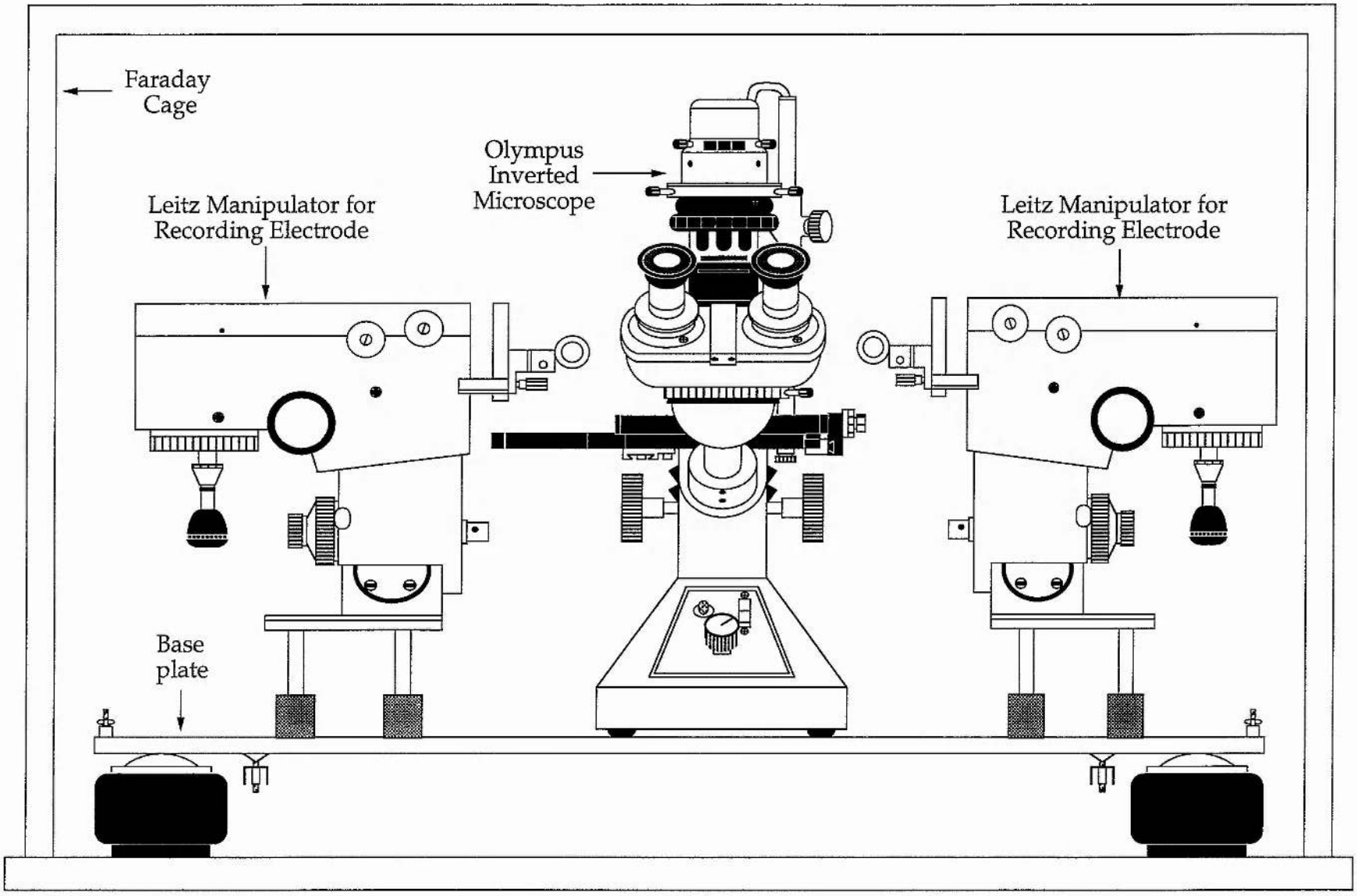
For all experiments on the B5 and B19 neurones a Digitimer NeuroLog NL 102 pre-amplifier was used. The other pre-amplifier used was an in-house built D.C. pre-amplifier. Signals from the neurones were passed to the pre-amplifiers via a Ag/AgCl wire. The apparatus is shown in figure 4. The signals from the pre-amplifiers were passed to a Racal Recorders 4 DS tape recorder, where they were recorded onto Ampex four channel magnetic tape, and then to a Tektronix 5113 dual beam storage oscilloscope. Hard copies of recordings were obtained on a Gould brush 220 pen recorder, the frequency response of which was 100 Hz . The frequency response of the Racal tape recorder was 1.25 kHz.

In some of the GDN / LSN experiments, two in-house pre-amplifiers were used. For the three cell experiments the Digitimer was used in conjuction with the two in-house units. In these two latter sets of experiments, the rest of the recording set-up remained as before. All of the pre-amplifiers used for recording were fitted with bridge circuits. Current injections were in the range of 0.5-3 nA.

The microscope, their lights and the manipulators were housed in a copper Faraday cage with a steel baseplate. Figure 4 shows the set-up.

Figure 4.

Diagram of the apparatus used during electrical recording from the isolated neurones. The Olympus inverted microscope shown replaced a Zeiss inverted microscope.


Faraday
Cage
Olympus
Inverted
Microscope
Leitz Manipulator for
Recording Electrode
Leitz Manipulator for
Recording Electrode
Base
plate

2.9 SOLUTIONS.

2.9.1 *Helisoma* Antibiotic Saline.

The standard antibiotic saline used in these experiments for sterilising animals and poly-L-lysine plates consisted of; 51.3 mM NaCl, 1.7 mM KCl, 1.5 mM $MgCl_2$, 4.1 mM $CaCl_2$ and 10 mM HEPES. To this was added 150 μg/ml gentamicin (Gibco), and the resultant saline was adjusted to pH 7.4 with 10 M NaOH.

The saline was filtered under suction through a 500 ml Nalgene filter unit fitted with a 0.2 μm cellulose-acetate filter (Sartorius). Saline was placed into sterile autoclaved bottle which were flamed, closed and sealed with PVC tape before being stored in the refrigerator.

This procedure was carried out under sterile conditions in the laminar flow hood.

2.9.2 *Helisoma* L-15 (Leibovitz) Defined Medium.

L-15 medium (Gibco), was supplied containing: D (+) galactose (900 mg/l), phenol red (10 mg/l), sodium pyruvate (550 mg/l), DL-α- alanine (450 mg/l), L-arginine (500 mg/l), L-asparagine (250 mg/l), L-cysteine (120 mg/l), glycine (200mg/l), L-histidine (250 mg/l), DL-isoleucine (250 mg/l), L-leucine (125 mg/l), L-lysine (75 mg/l), DL-methionine (150 mg/l), DL-phenylalanine (250 mg/l), L-serine (200 mg/l), DL-threonine (600 mg/l), L-tryptophan (20 mg/l), L-tyrosine (300 mg/l), DL-valine (200 mg/l), DL-Ca pantothenate (1 mg/l), choline chloride (1 mg/l), folic acid (1 mg/l), i-inositol (2 mg/l), nicotinamide (1 mg/l), pyridoxine HCl (1 mg/l), riboflavin-5′-phosphate, sodium (0.1 mg/l), thiamine monophosphate (1 mg/l).To this basic medium salts were added; 51.3 mM NaCl, 1.7 mM KCl, 1.5 mM $MgCl_2$, 4.1 mM $CaCl_2$ and also 10 mM HEPES buffer. Glutamine (48 mg/100ml) was also added as was D-glucose (6 mg/100ml). The solution was then made up to the required volume with sterile milli Q water. Gentamicin (Gibco) was added at 100μg/ml, and the medium was adjusted to pH 7.4 using 10 M NaOH.

The medium was filtered under suction through a 250 ml Nalgene filter unit fitted with a 0.2 μm cellulose-acetate filter (Sartorious). When filtration was completed the medium was dispensed into plastic culture flasks (Cel-Cult or Corning), and stored in the refridgerator.

2.9.3 Tris Buffer.

The Tris buffer used in the preparation of the poly-L-lysine was made up as follows; Trizma HCl and Trizma base (both Sigma) were dissolved in 100 ml of milli Q water to pH 8.4 at 25°C to give a final concentration of 0.15 M. The solution was then filtered through a 0.2μm cellulose-acetate filter (Sartorious) fitted in a Nalgene 250 ml filter unit, and placed into a sterile autoclaved bottle.

CHAPTER 3
RESULTS

3.1 CULTURE CONDITIONS.

Neurones were placed in culture under various conditions and the amount of neuritic growth produced from them noted. The culture conditions used were; 1. Defined medium with unconditioned poly-L-lysine (26 kDa MW); 2. Defined medium with conditioned poly-L-lysine (26 kDa MW); 3. Conditioned medium with unconditioned poly-L-lysine (26 kDa MW).

Process extension was classified as follows; no neurite extension, neurite extension less than the diameter of the neurone and neurite extension greater than the diameter of the neurone. This procedure was chosen since it has been reported previously (Wong *et al,* 1981).

The results from these experiments are shown in table 1 and graphically in figure 5. It can be seen from the graph that neurones placed into culture in defined medium alone showed no, or very little, neurite extension. The use of conditioned medium was seen to greatly increase neurite extension from the isolated neurones.

3.2 EXPERIMENTS WITH NEURONES B5 AND B19.

It had been previously demonstrated that the B5 and B19 neurones, from the buccal ganglia of *Helisoma* , were capable of forming a unidirectional cholinergic connection in cell culture (Haydon, 1988). This connection had been shown to be from the B5 cell to the B19. These neurones were therefore chosen for the initial studies, with a view to using them as the basis for constructing a circuit at a later date.

Figure 6 shows the position of the B5 and B19 neurones within the buccal ganglia of *Helisoma* . The B5 neurone was 55-60 μm in diameter and had only one axon, whereas the B19 neurone had two axons and was slightly smaller at 45-50 μm. Each B5 neurone sends an axon ipsilaterally out of the buccal ganglion via the oesophageal nerve, from which it enters the duct of the salivary glands. B19 projects one of its axons ipsilaterally out of the buccal ganglia and the other contralaterally. This neurone sends its axonal projection from the ganglia to the buccal musculature via the ventrobuccal nerves. The B19 innervates the supralateral tensor muscle, which plays a part in protracting the radula during feeding (Kater, 1974).

Table 1

Table showing the data from the experiments on neurite extension. Figures were calculated using B5 and B19 neurones under the various culture conditions. These data are shown graphically in figure 5.

	DEFINED MEDIUM	CONDITIONED PLATES DEFINED MEDIUM	CONDITIONED MEDIUM
NO EXTENSION	21	13	2
EXTENSION LESS THAN DIAMETER OF CELL BODY	8	18	9
EXTENSION GREATER THAN DIAMETER OF CELL BODY	0	4	15
TOTAL CELLS	29	35	26

Figure 5.

Graph showing percentage of neurones extending neurites under various culture conditions. DM.= Defined Medium. COND. DM.= Conditioned plates and Defined Medium. CM.= Conditioned Medium.

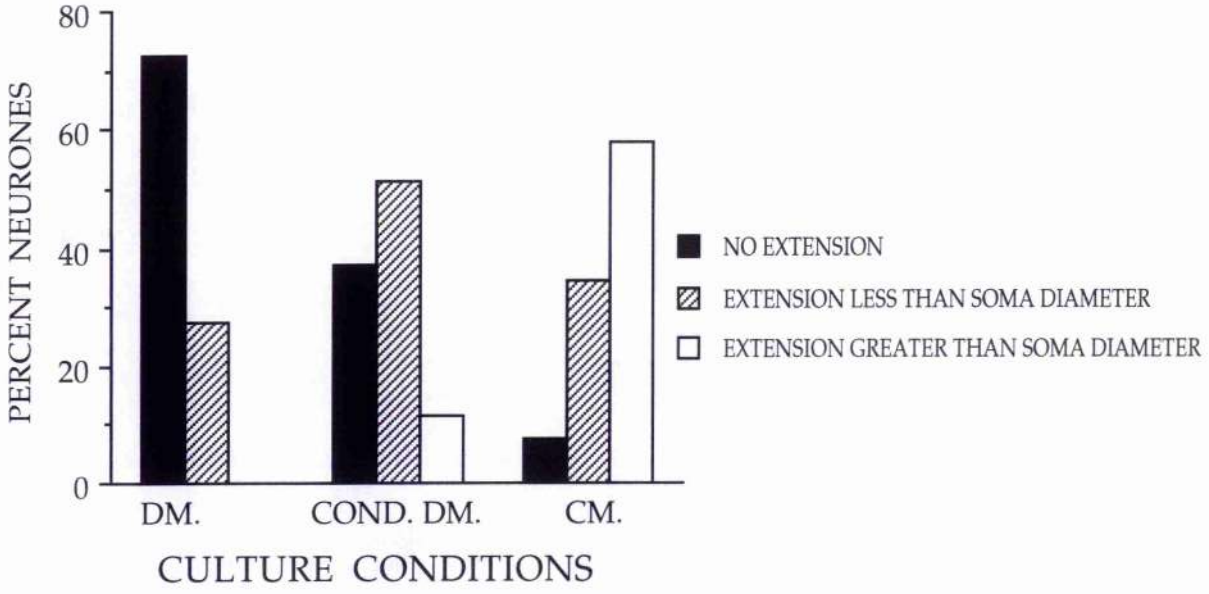
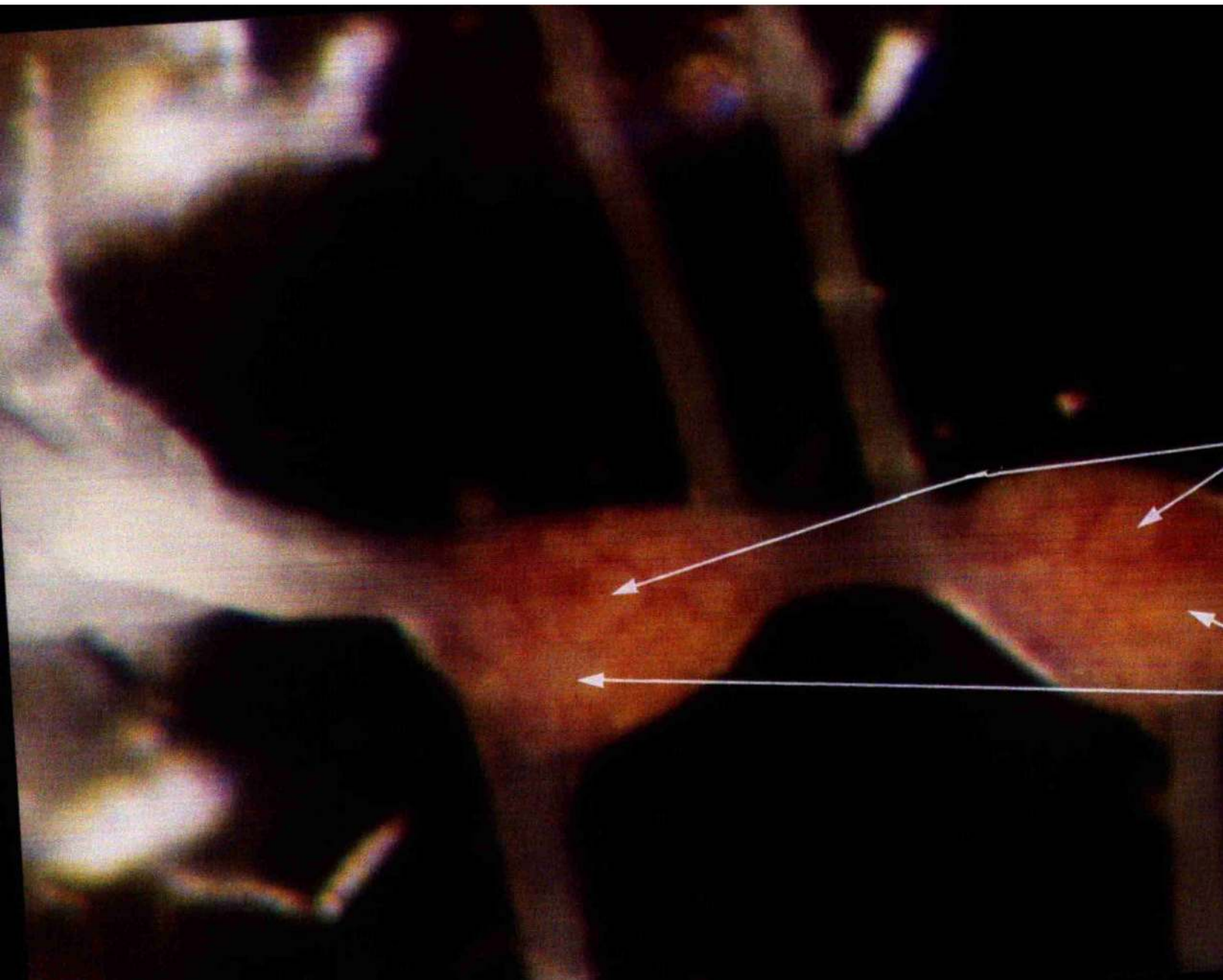

80
60
40
20
0
PERCENT NEURONES
DM.
COND. DM.
CM.
CULTURE CONDITIONS
NO EXTENSION
EXTENSION LESS THAN SOMA DIAMETER
EXTENSION GREATER THAN SOMA DIAMETER

Figure 6.

Photograph of the paired buccal ganglia of *Helisoma trivolvis*. The positions of the B5 and B19 neurones are marked by arrows. Each B5 is marked with long arrows and the B19 neurones with short arrows. Scale bar= 1 mm.



3.2.1 Soma-Soma Contacts.

The work by Haydon (Haydon, 1988) had demonstrated that it was possible to obtain chemical junctions between the cell bodies of two isolated neurones after three days in culture conditions. Initial experiments involving the B5/B19 pairing were therefore concentrated on trying to obtain connections between the neuronal somata. The neurones were placed in cell culture conditions that were unfavourable to the formation of neurites for these experiments. These conditions were defined medium with unconditioned poly-L-lysine substrate. Under these conditions 72% of neurones failed to produce any neurites (see figure 5).

To obtain soma-soma contact between the two neurones, one of the cells was placed into the culture dish and left for 20 minutes. During this period the neurone adhered firmly to the substrate. The second neurone was then added so that the somata of the two cells were in contact. Gentle positive pressure was then applied to the pair from the suction pipette to make sure that the somata were firmly apposed. The pair of neurones were then left undisturbed for a further 20 minutes before the culture dish was moved. This period was necessary to ensure that the neurones were properly adhered to both the substrate and each other. Figure 7(a) shows an example of a B5/B19 pair placed in culture using this technique. The neurones in this figure had been in culture conditions for 20 hours.

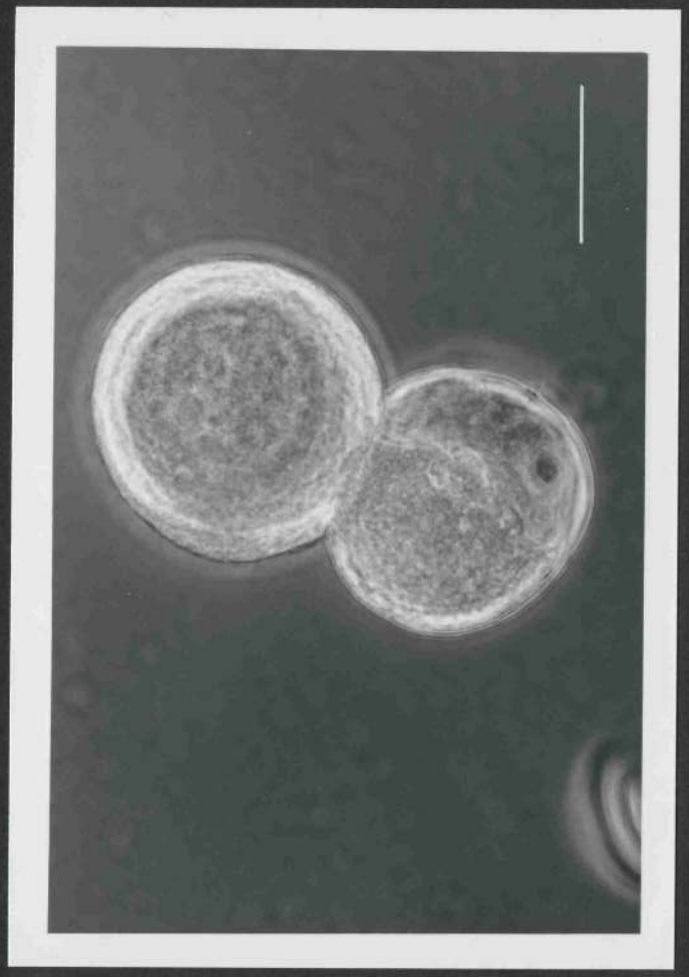
Neurite extension from neurones in these culture conditions occured in only 28% of such pairs. Areas of veiling could often be observed around the somata however. These areas resembled large growth cones in their appearance, consisting of lamellipodia and filopodial processes. They often completely surrounded a single neurone. Such extensions were present after 15-18 hours of isolation, and often remained visible for two to three days. These structures are shown in figure 7(b). Neurite extension from these areas was rarely seen. Any neurites produced by neurones exhibiting this type of veiling were never longer than the diameter of the cell itself.

Electrophysiological recordings (n=10) were made from B5/B19 pairs

Figure 7.

(a) Photograph showing B5 (bottom) and B19 (top) forming a soma-soma contact in culture conditions which discourage neurite extension. The membranes of the two neurones appear fused, and no neurite outgrowth can be observed. No connection was detected between these neurones. Scale bar= 30 ≡m.

(b) B5 (right and left) and B19 (middle) in similar conditions to those in (a). Areas of filopodial and lamellipodial extension can be clearly seen around these neurones with no neurites being present. The soma of these neurones are in close contact as in (a). An electrical connection was recorded between the left- hand B5 and the B19 neurones of this trio. This connection is shown in figure 8 (a). Scale bar= 50 ≡m.



using microelectrodes filled with 1M KCl after the neurones had been in culture for two to four days. Connections were studied with the proposed postjunctional neurones active and quiescent. Membrane potentials in proposed postjunctional neurones were altered by the application of bias currents to the neurones from the recording electrode.

The experiments did not reveal any chemical connections between these neurones. All junctions between the neuronal cell pairs were found to be electrical in nature (n=2). Electrical connections were determined by their ability to pass hyperpolarising current from one neurone to another. In these two cases both hyperpolarising and depolarising current injections to the prejunctional neurone were communicated to its partner. The neurones were therefore connected by bidirectional electrical junctions. The first of these connections was detected on the second day after isolation and the other on the third day. Examples of these recordings are shown in figure 8. No connection was detected between the neurones in the remaining eight cases, despite the neurones appearing to make contact with one another.

3.2.2 Contacts Via Neurite Interaction.

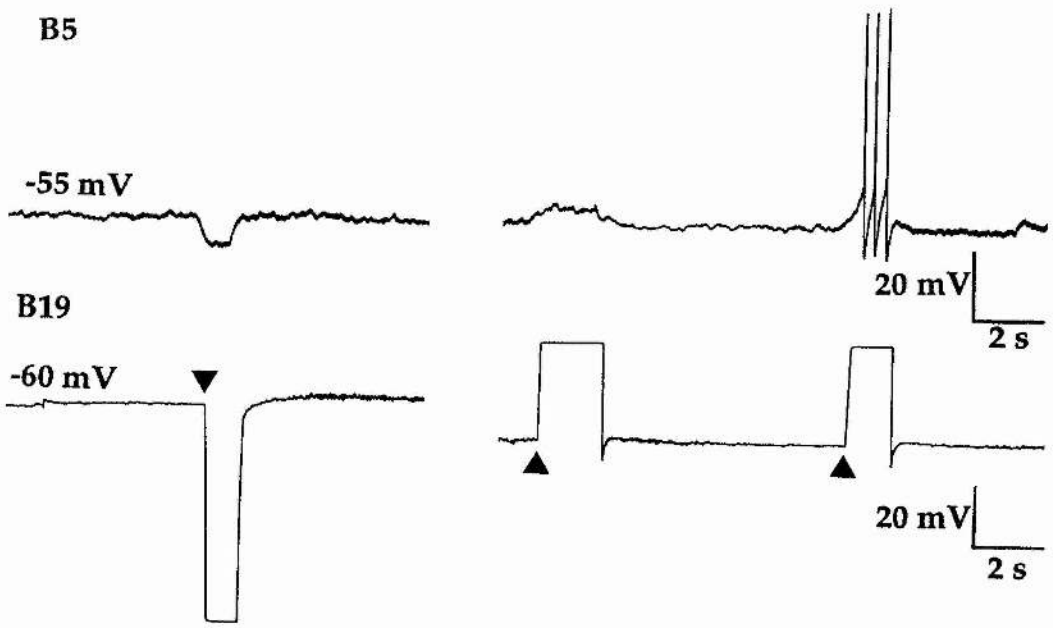
Due to the lack of success in obtaining chemical connections using the soma-soma method, a change was made to the culture conditions. This alteration was designed to try to form chemical junctions between neurones that extended neurites. Connections between neurones extending neurites has previously been obtained by several groups using neurones isolated from *Lymnaea stagnalis* (Syed *et. al,* 1990), *Aplysia californica* (Camardo *et. al,* 1983) and *Hirudo medicinalis* (Ready and Nicholls, 1979).

The culture conditions used in these experiments were 26 kDa MW poly-L-lysine substrate and 2ml of conditioned medium rather than defined medium. Under these conditions 58% of neurones produced neurites that were greater in length than the diameter of the perikaryion (see graph in figure 5). The majority of this extension occurred by 15-18 hours after isolation. This allowed neurones placed close together in the culture dish to extend neurites which could overlap to give contact. Figure 9 shows a photograph (top print) of B5 and B19 neurones under these neurite promoting conditions showing that extensive neuritic

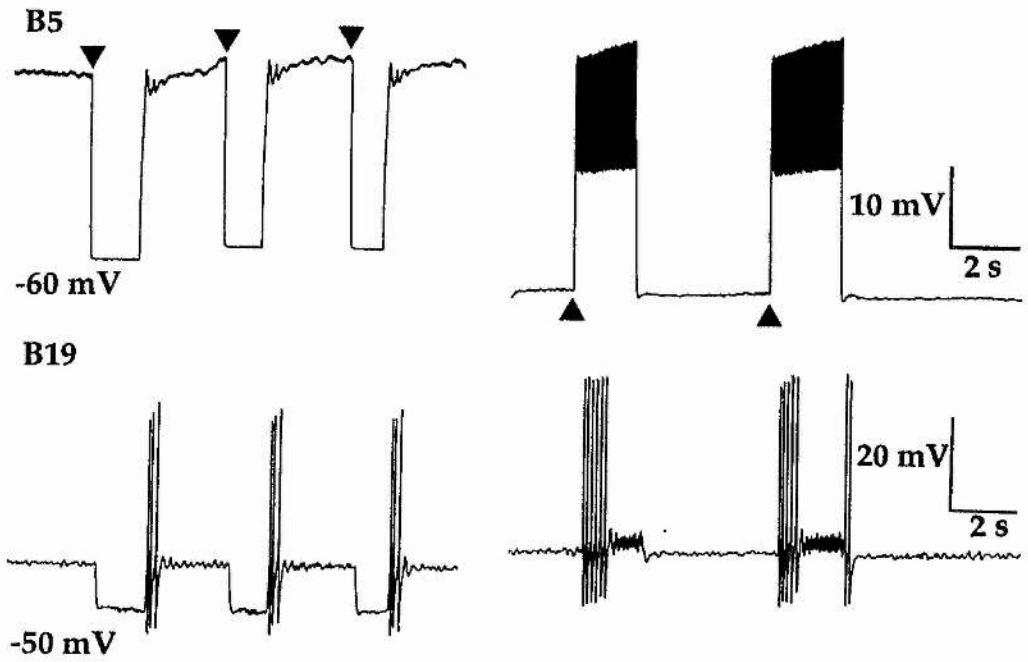
Figure 8.

(a) An electrical connection obtained from a B19 neurone to a B5 on the second day after isolation. Injections of both hyperpolarising and depolarising D.C. current into the B19 neurone are communicated to the B5 neurone, suggesting that this junction was non-rectifying. Current injections were made at the points marked by arrowheads. Both neurones at resting membrane potential, which are indicated by the voltages shown. Action potentials in this, and all other recordings, were truncated by the chart recorder used. The neurones used in this experiment are shown in the photograph in figure 7 (b).

(b) Electrical recordings obtained from B5 and B19 neurones after three days in culture showing a reciprocal electrical connection. Hyperpolarising and depolarising current injections into the B5 neurone are communicated to the partner. As in (a) the passage of current of both signs indicates the presence of a non-rectifying electrical junction. Points where injections of current were made are marked by arrowheads. Both neurones at resting membrane potential as indicated.


B5
-55 mV
20 mV
2 s
B19
-60 mV
20 mV
2 s

(a)


B5
-60 mV
10 mV
2 s
B19
-50 mV
20 mV
2 s

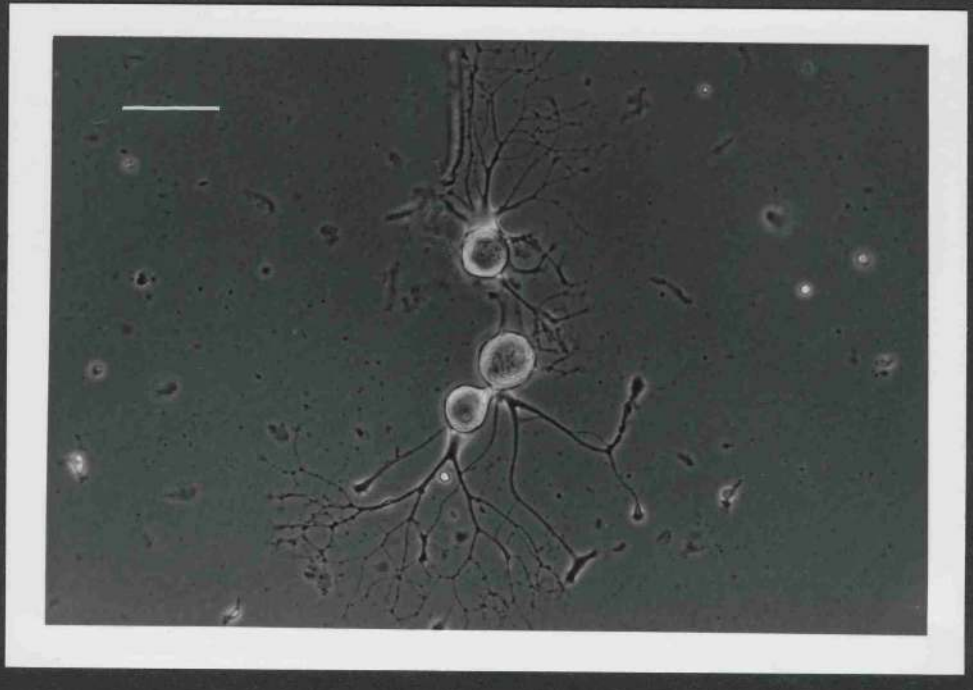
(b)

Figure 9.

Photographs showing B5 and B19 neurones placed in culture in conditions promoting neurite extension.

Upper plate. Neurones in 2 ml CM. with 26 kDa substrate after one day in culture. This photograph shows the extent of neurite growth that could be achieved after one day in culture. Two B19 neurones are shown (top and bottom) with one B5 neurone (middle). Electrical connections were made from these neurones on days two and three after isolation. An electrical connection was detected between the top B19 and the B5 on day three. The electrophysiological characteristics of this connection are shown in figure 10. Scale bar=100 μm.

Lower plate. Neurites from the bottom B19 neurone. The neurites are thicker toward the soma and gradually become thinner towards the growth cones. Small varicosities are visible at points along the length of some the neurites. Scale bar= 20μm.



growth that can occur under these conditions. The neurites showed a high degree of branching and became noticeably thinner towards the terminus, each of which was capped by a growth cone. Growth cones were characteristically flat in appearance, with finger-like filopodial processes stretching out from them (figure 9, lower print). The characteristics of such growth cones changed with time, becoming noticeably smaller and more rounded with very long fine filopodia when they stopped extending, usually after day three in isolation. After this the growth cones became rounded and phase bright, with no filopodia. This situation was always seen after four days in culture conditions. Neurites were capable of being extended from the neurone in any direction, indicating that the axon hillock was not the only part of the neuronal membrane that could produce neurites.

Intracellular recordings (n=6) from these neuronal pairs were made after they had been in culture conditions for two to four days, as before. In only one of the pairs was a connection detected. This was a very weak electrical connection from the B5 to the B19 which was detected on day three of culture. Both depolarising and hyperpolarising current was communicated from the B5 to the B19 in this case. No connection was detected from the B19 to the B5 (data not shown). Recordings from this pair of neurones are shown in figure 10 (a).

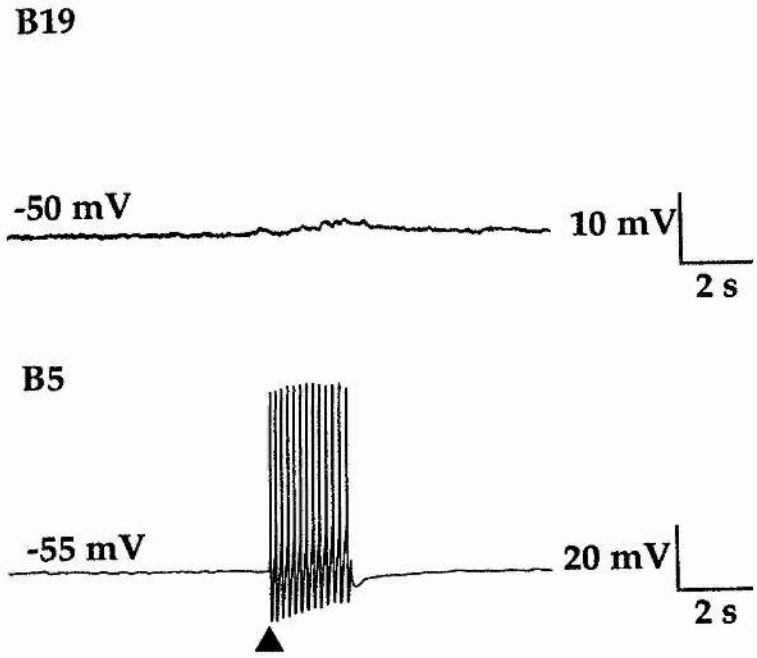
In one of the remaining five pairs what appeared to be spontaneous excitatory synaptic potentials were observed in the B19 neurone, with no corresponding activity in the B5. These fast events were detected at several points during the recording of activity in this B19 neurone. No junction, either electrical or chemical, was detected between this B5/B19 pair. An example of these possible excitatory synaptic potentials are shown in figure 10(b).

Since no chemical junctions had been detected under these conditions, the culture conditions were again altered. The change that was made involved replacing the 26 kDa MW poly-L-lysine with 10 kDa MW poly-L-lysine. The neurones were still able to grow on this substrate (see figure 11), producing neurites which tended to be longer and appeared thinner than those produced on the 26 kDa MW substrate. The neurites extended on 10 kDa MW poly-L-lysine were also observed to branch less frequently than those on the higher MW substrate.

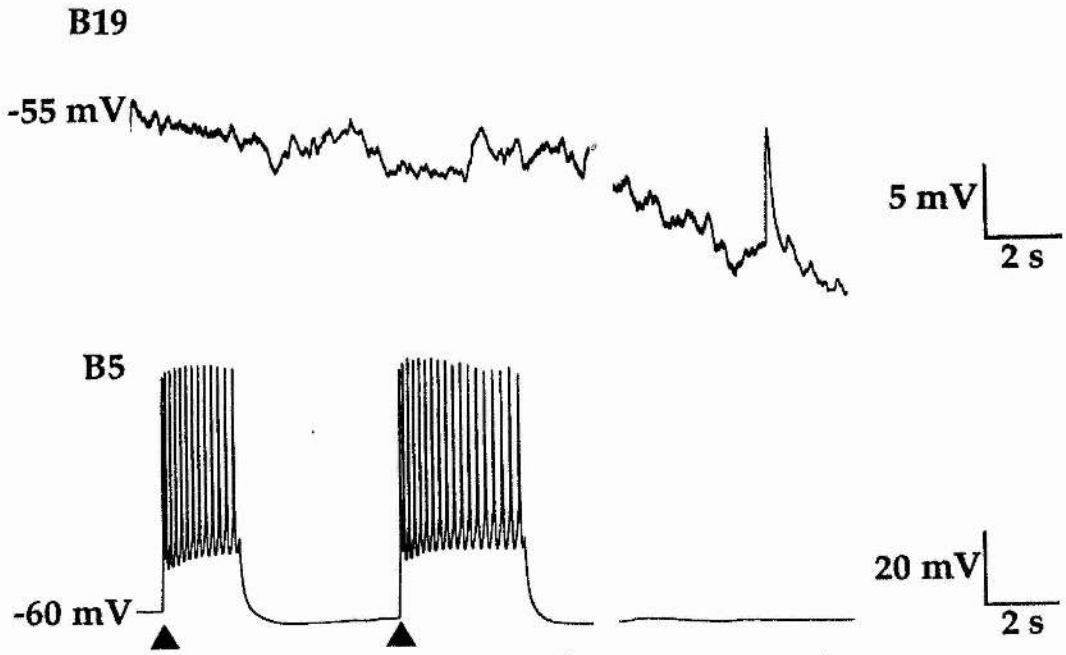
Figure 10.

(a) Weak electrical connection detected between a B5 and B19 neurone where both neurones were extending neurites. These neurones were isolated into 2 ml conditioned medium with 26 kDa. poly-L-lysine substrate. The recording shown was made on day three after isolation. Depolarising current was injected into the B5 neurone at the point indicated by the arrowhead. Hyperpolarising current was also passed from the B5 to the B19 (not shown). No responses were observed from the B19 to the B5 (not shown). The passage of both depolarising and hyperpolarising current from the B5 to the B19 suggests that the neurones were connected by a bidirectional electrical connection. Both neurones at their respective resting membrane potential which are indicated on each recording. The neurones used in this experiment are shown in the photograph in figure 9.

(b) Recordings made from a B5/B19 pair under identical conditions to those in (a). In this case no connection was detected between the neurones (left) but activity which resembled excitatory synaptic potentials could be recorded in the B19 neurone despite no activity in B5 (right). B19 at resting membrane potential (shown on recording). Hyperpolarising current was injected into B5 to prevent firing of action potentials whilst the neurone was at resting potential (-55 mV).



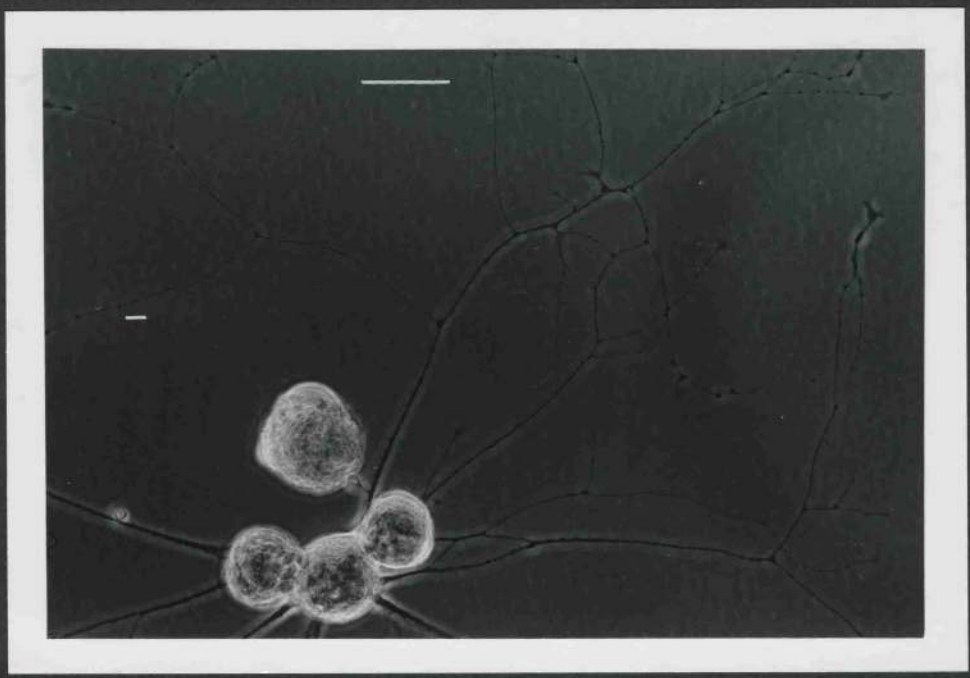
(a)



(b)

Figure 11.

Photograph of B5 (middle) and B19 (left and right) on 10 kDa poly-L-lysine substrate with 2ml conditioned medium one day after isolation. The fourth neurone present was a B5 which was non-viable. The neurites produced here are very long and thin with fewer branches than those shown in figure 9. The neurites are slightly thicker near the somata than at the growth cone. Varicosities are present at a few places along the neurites. Scale bar = 50 μm.



Recordings (n=7) were made under these conditions after only one day in isolation, and on subsequent days until the neurones were no longer viable. In only one case was a connection recorded. This was a weak electrical connection recorded on day two in culture, and is shown shown in figure 12. The figure shows that hyperpolarising current was passed in both directions. No connection was present between this cell pair on day one. In the remaining cases however, no connections were detected between the B5/B19 pairs under these culture conditions (n=6).

3.3 EXPERIMENTS WITH THE GIANT DOPAMINE NEURONE (GDN) AND THE LARGE SEROTONIN NEURONE (LSN).

Since the experiments to obtain chemical connections with the B5 and B19 pairs were unsuccessful, experiments were carried out on another pair of neurones. The pair of neurones which were chosen were the giant dopamine neurone, GDN, and large serotonin neurone, LSN, from the left and right pedal ganglia respectively. These neurones were chosen because chemical junctions had previously been obtained between them in the laboratory (Stuart Harris, unpublished observations).

The positions of these neurones within the central ganglia of the animal are displayed in figure 13.

The GDN was found to be about 70-80 μm in diameter. This neurone projects a single axon out of the left pedal ganglion into the left pleural and parietal ganglia. The axon branches in the left parietal ganglion, and one of the branches passes out of the central ring ganglia via the left internal and external parietal nerves. The second branch passes into the visceral ganglion and left the central ring for the periphery via the anal nerve (Syed *et. al,* 1993).

The LSN soma, which was 60-70 μm in diameter, was observed to be the mirror image of the GDN. The single axon from the LSN passes out of the right pedal ganglion into the right pleural and parietal ganglia. In the right parietal ganglion the axon branches. One of the branches leaves the central ring via the right parietal nerve, whereas the remaining branch passes into the visceral ganglion which it leaves, like the GDN, through the anal nerve. Both the GDN and LSN display fine processes in the pedal ganglia. There is no evidence that either of these neurones

Figure 12.

Electrical recordings from a B5/B19 pair isolated into 2 ml conditioned medium with 10 kDa. poly-L-lysine substrate. A weak bidirectional electrical connection was detected between these neurones on the first day after isolation.

(a) Hyperpolarising current injected into neurone B5 was communicated to the B19 neurone. The B19 neurone was at resting membrane potential. The B5 neurone was hyperpolarised to prevent firing of action potentials at resting membrane potential (-50 mV). Current injection indicated by arrowheads.

(b) Hyperpolarising current injections into the B19 neurone were passed to the B5 neurone. Both neurones were hyperpolarised from their resting membrane potentials. Current injections to the B19 are marked by the arrowheads.

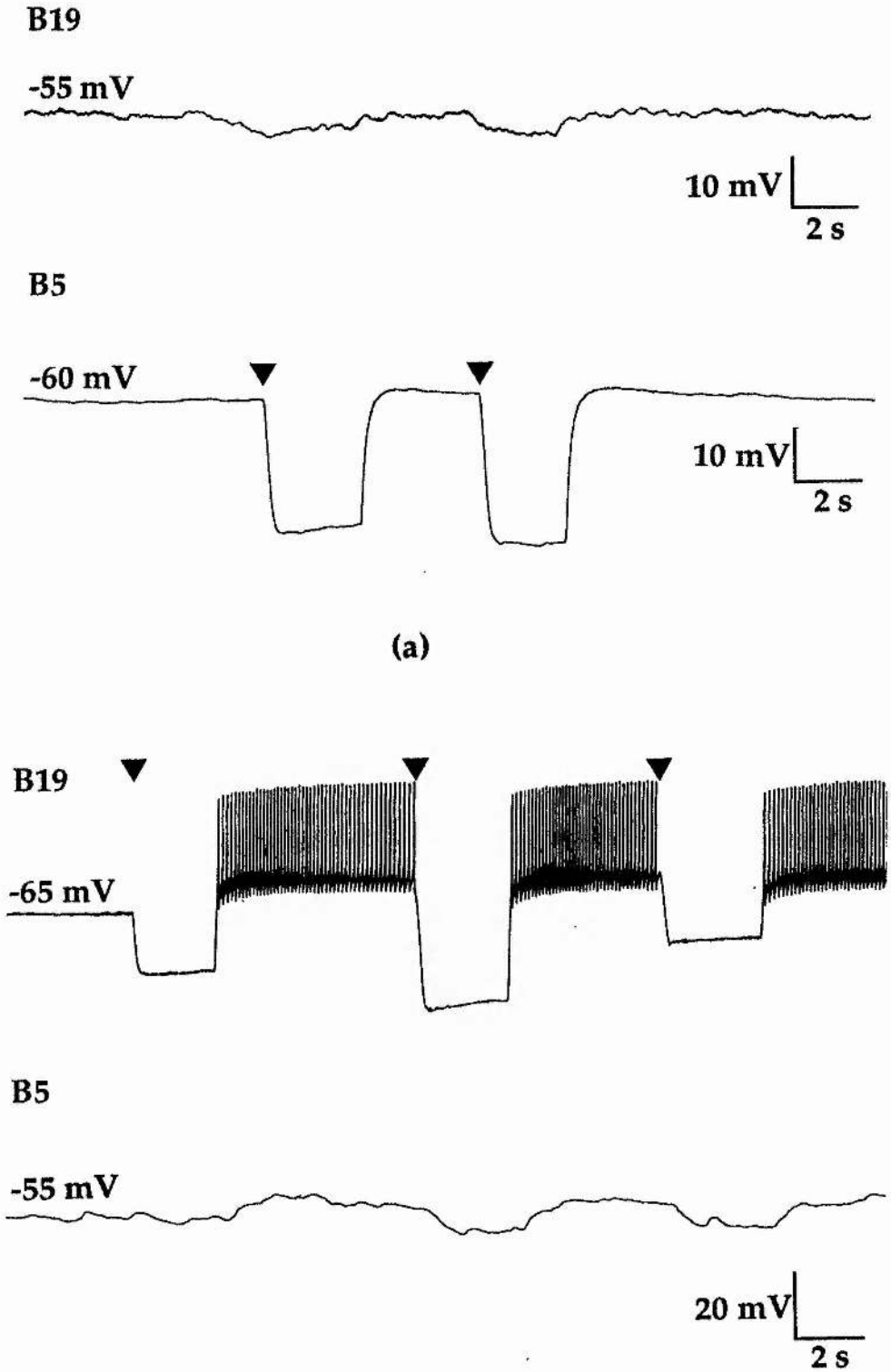

B19
-55 mV
10 mV
2 s
B5
-60 mV
10 mV
2 s
(a)
B19
-65 mV
B5
-55 mV
20 mV
2 s
(b)

Figure 13.

Photograph of the central ganglia showing the positions of the LSN and GDN in the pedal ganglia. The GDN is marked with a long arrow and the LSN with a short arrow. Scale bar= 100 μm.

innervates the contralateral pedal ganglion. Diffuse processes are also present from the two pedal neurones in the pleural, parietal and visceral ganglia (Syed *et. al,* 1993).

Both the GDN and LSN had very prominent areas of reddish-orange pigment near their axon hillocks. This pigmentation, coupled with the large size and constant position of the GDN and LSN within the pedal ganglia, aided their identification.

3.3.1 Contacts Via Neurite Interaction.

The experiments with the GDN/LSN pairs were carried out with conditioned medium on 10 kDa MW poly-L-lysine. Under these conditions the neurones extended long thin neurites which were capable of overlapping with each other. Photographs of GDN/LSN pairs under these conditions are shown in figures 14 and 15. Typically a large amount of the neurite outgrowth occurred during the first 15-18 hours (see figures 14 and 15), which allowed electrophysiological experiments to be carried out on the neuronal pairs on the first day after isolation (day one). Recordings could then be made on subsequent days until the neurones were no longer viable.

Neurite extension from these pairs was seen to be mainly from the axon stump, with other areas of the soma membrane showing little or no extension. However, in cases where such extension was apparent, the neurites seemed most likely to be produced from the part of the membrane opposite the axon hillock. The neurones were plated with their somata close together and their axons overlapping, often more than once.

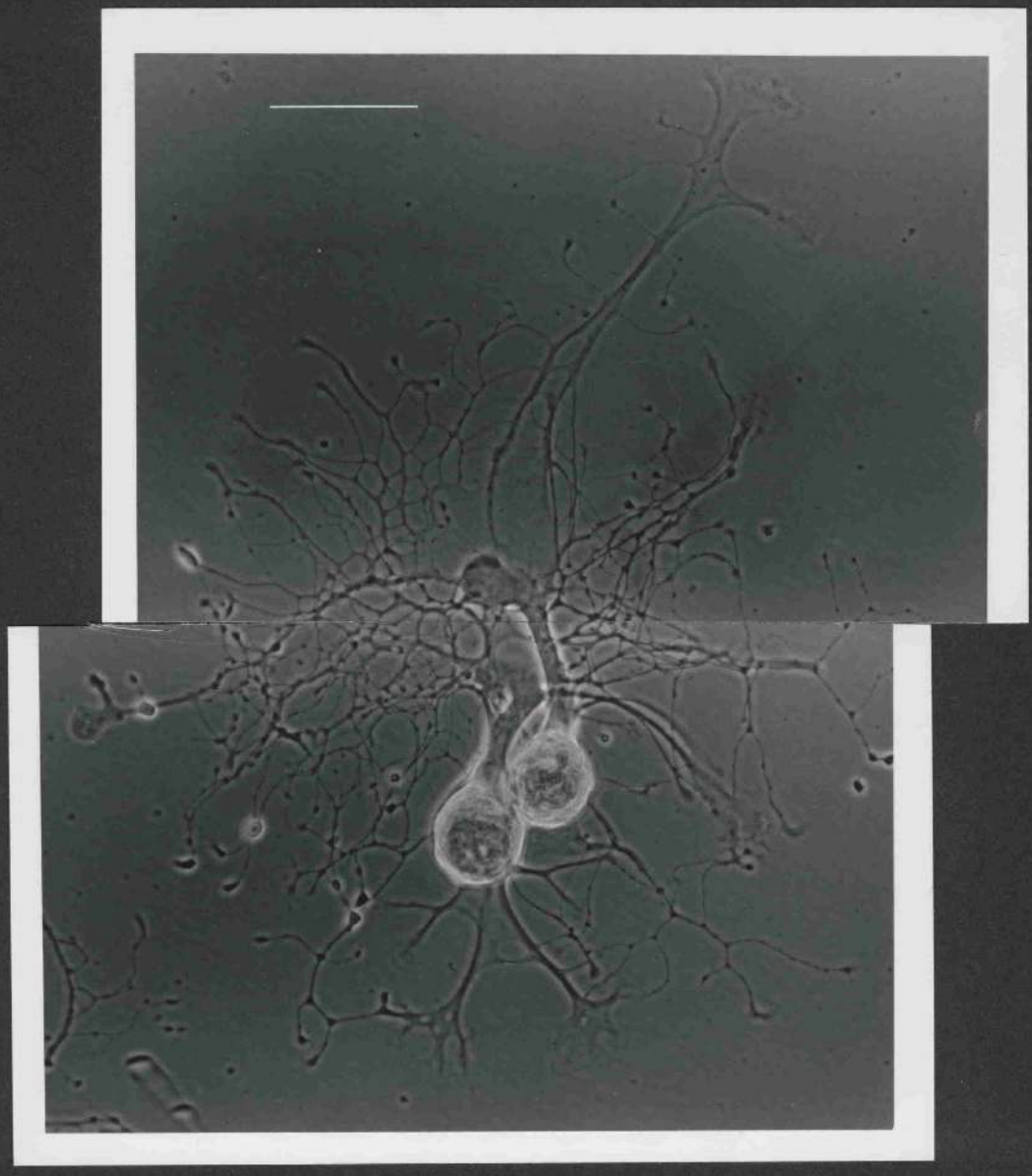
The GDN/LSN pairs often made inhibitory chemical connections (n=12). The inhibitory connections between these neurones were determined as being chemical if they could be abolished by altering the postjunctional membrane potential. In addition to this hyperpolarising current was not passed between the neurones. These connections were unidirectional, with reciprocal connections never being observed. Injection of depolarising current into the prejunctional neurone lead to a burst of action potentials in this cell. This in turn gave rise to a hyperpolarisation in the follower neurone which was slow in onset and

Figure 14.

Photograph of a GDN/LSN pair after one day in culture. LSN (top) and GDN (bottom). The amount of neurite outgrowth and overlap possible between these neurones in this space of time was comparible to that obtained between the B5 and B19 in figure 9. This photograph shows how the neurones were placed in culture, with their axons overlapping and somata in contact. Electrical recordings were made from these neurones and revealed the presence of an inhibitory chemical connection from the LSN to the GDN. Scale bar= 100 μm.

Figure 15.

A GDN (right) and LSN (left) after one day in culture. These neurones displayed even more extensive neurite outgrowth and overlap than those in figure 14 over a similar timescale. No connection was detected between these neurones when recordings were made from them over a three day period. Scale bar= 100 μm.



long-lasting. Both the GDN to LSN connections and those from the LSN to the GDN were abolished by hyperpolarising the follower neurone to -80 mV. These results are shown in figures 16 and 17. Chemical connections were most likely to form from the LSN to the GDN (n=9), with GDN to LSN connections forming less often (n=3). On many occasions electrical connections were detected between GDN/LSN pairs. These connections were always reciprocal (n=9). Mixed chemical and electrical connections were also recorded (n=5). These connections were determined as those which were able to pass hyperpolarising current and had a component that was abolished by altering the postjunctional membrane potential. Examples of electrical and mixed connections detected are shown in figure 18. All of the electrical connections detected were bidirectional. Connections were not observed in the remaining cases (n=14). These figures are calculated from the connection first detected between a neurone pair. Altogether recordings were made from forty GDN/LSN pairs.

Recordings were made from pairs of neurones over a period of three days. By studying these recordings it was possible to see how the connections progressed over the recording period. These observations are shown in table 2. From these data there appears to be a progression from a junction that is chemical in nature during the early stages of isolation to one that is electrical. This change from one type of connection to another seems to occur gradually via an intermediate stage. This intermediate stage appears to be a mixed form of connection in which both electrical and chemical elements are present.

When recordings from pairs were treated separately this gradual change in the nature of the connections was only seen in one case. Many of the mixed junctions were detected on day two in culture, but three of these five connections were recorded from neurones that had not been in contact on day one. When recordings were made from these pairs after three days in culture, reciprocal electrical connections were present in the two pairs that were still viable. This suggests that mixed junctions are likely to become electrical with time. Connections that progressed from chemical to electrical with no intermediate were not detected.

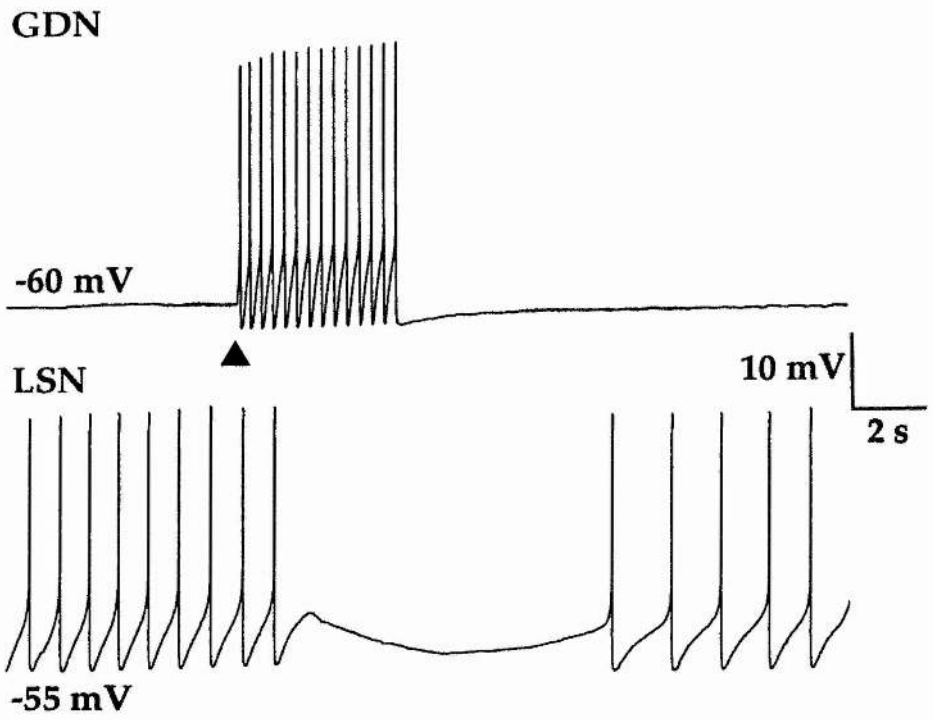
On two occasions the chemical connections were found to gradually weaken over two days until no connection could be detected. One of

Figure 16.

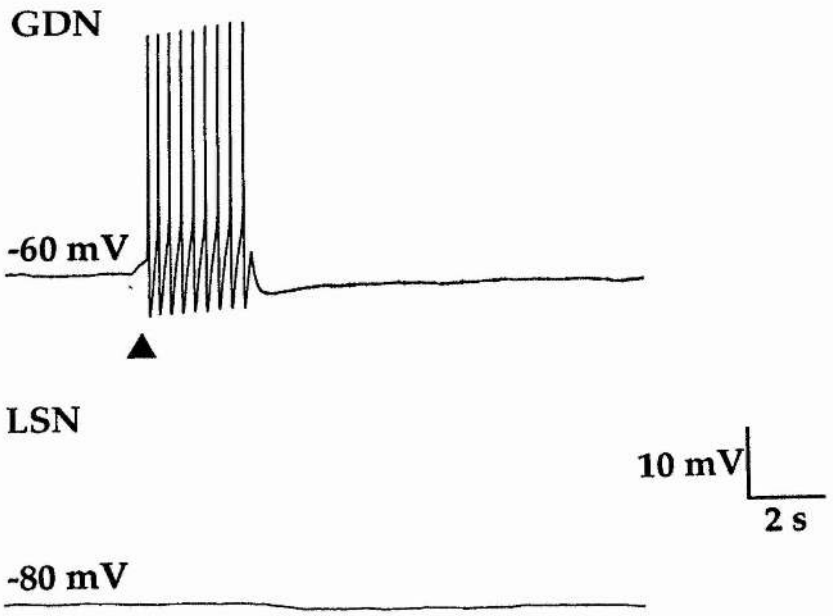
Electrical recordings from a GDN/LSN pair after one day in isolation.

(a) Injection of depolarising current into the GDN gave rise to a burst of action potentials, which caused inhibition of the activity in the LSN. The LSN was at resting membrane potential (shown on recording) whereas the GDN was hyperpolarised from resting potential (-55 mV) in order to prevent firing of action potentials. Depolarising current injection indicated by arrowhead.

(b) Recording showing the abolition of the response shown in (a). Hyperpolarising current was injected into the LSN to maintain its membrane potential at -80 mV. Depolarisation of the GDN no longer gave rise to the hyperpolarisation of the LSN membrane potential observed in (a). Depolarisation of the GDN indicated by the arrowhead.



(a)



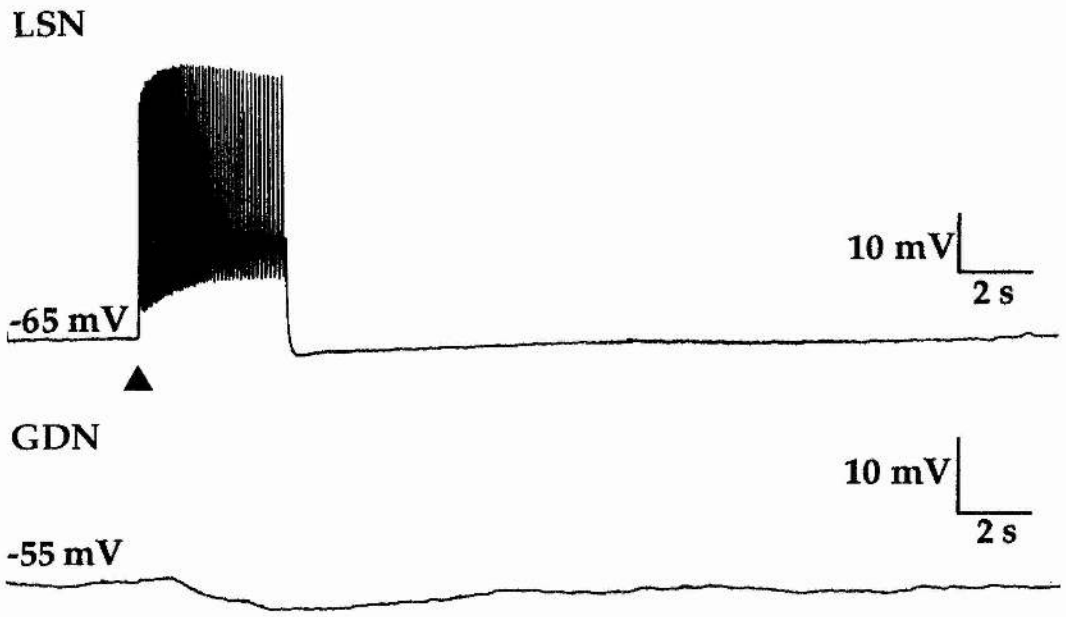
(b)

Figure 17.

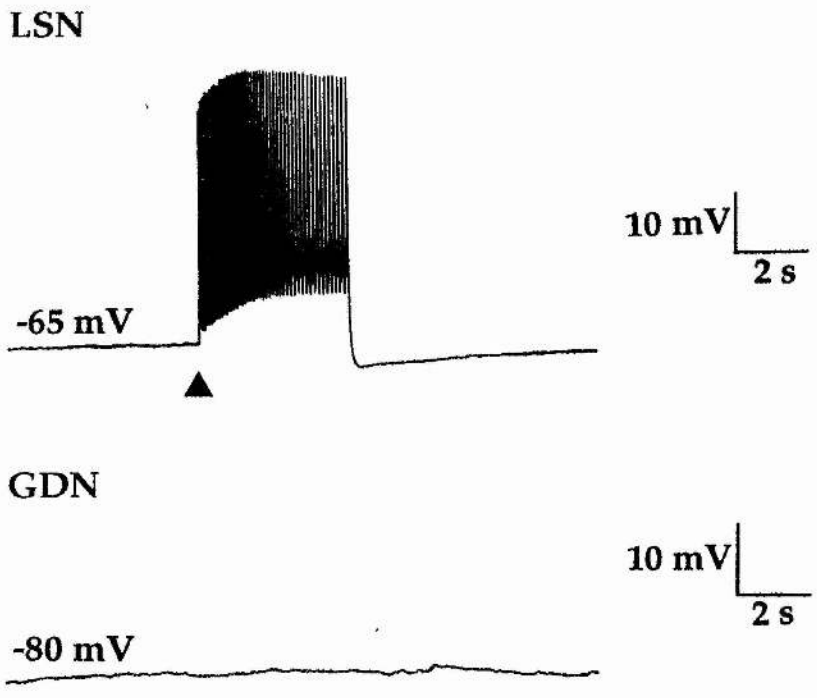
Recordings from a GDN/LSN pair after one day in isolation.

(a) A chemical connection detected from the LSN to the GDN. Injection of depolarising current into the LSN (marked by the arrowhead) gave rise to a burst of action potentials in this neurone. This burst caused a hyperpolarisation of the GDN membrane potential. Hyperpolarising current was injected into the LSN to keep the neurone below its threshold potential for action potential firing. The LSN had a resting membrane potential of -55 mV as did the GDN (shown on recording).

(b) Recordings of the abolition of the response shown in (a). Hyperpolarising current was injected into the GDN to maintain its membrane potential at -80 mV. Depolarisation of the LSN (marked by the arrowhead) no longer caused a hyperpolarisation of the GDN membrane potential. The GDN membrane potential as in (a).


LSN
10 mV
2 s
-65 mV
GDN
10 mV
2 s
-55 mV

(a)


LSN
10 mV
2 s
-65 mV
GDN
10 mV
2 s
-80 mV

(b)

Table 2

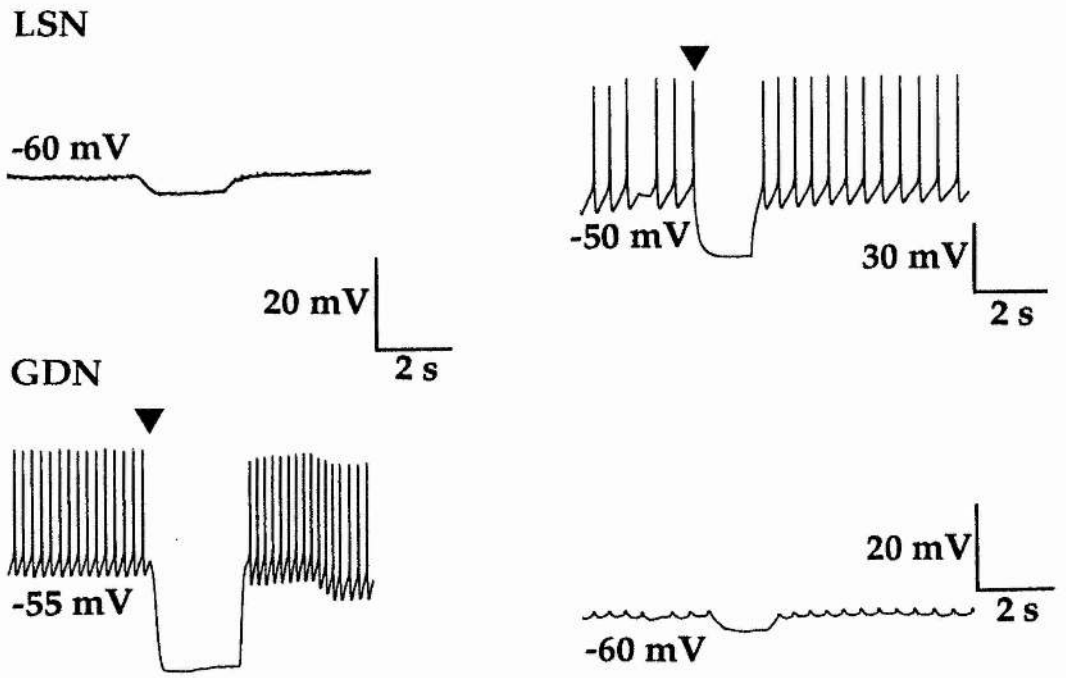
This table shows the progression of connections over a period of three days in culture. Figures are expressed as percentages. The table suggests that chemical connections become less likely to be present as time in culture increases. Electrical connections appear more likely to be present with increased time in culture. The table also shows that no connection between neurones was the most likely outcome at any stage.

	DAY 1	DAY 2	DAY 3
CHEMICAL	29.4	9.5	6.3
MIXED	2.9	23.8	0
ELECTRICAL	14.7	23.8	43.7
NO CONNECTION	52.9	42.9	50

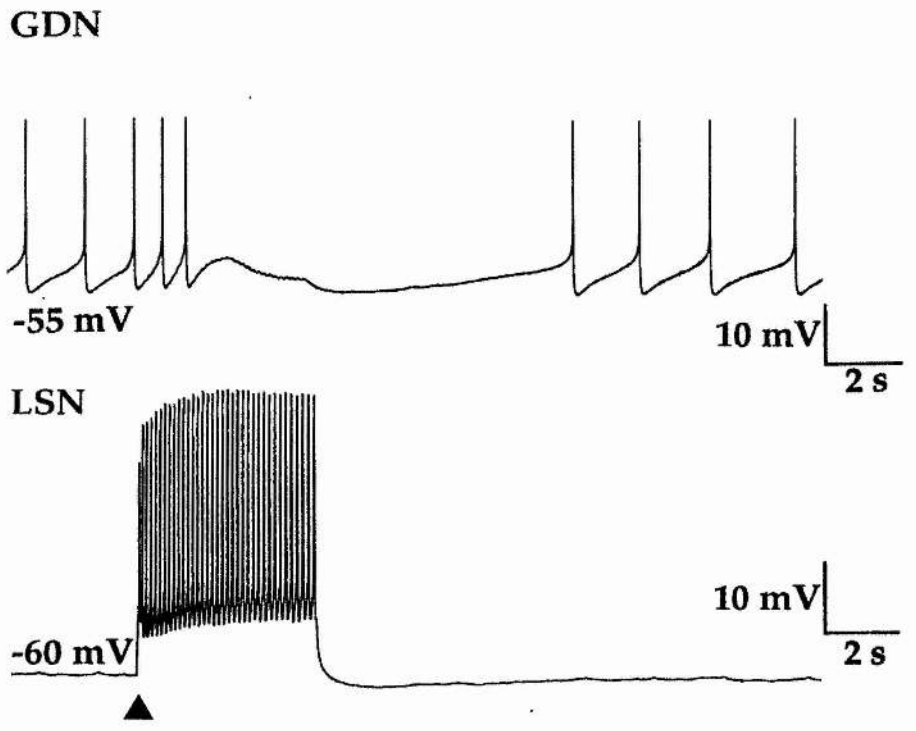
Figure 18.

(a) Electrical recordings showing a reciprocal electrical connection between a LSN and a GDN. Hyperpolarising current was communicated from the GDN to the LSN (left panel). The GDN was at resting membrane potential (shown on recording). The LSN was hyperpolarised from its resting membrane potential in order to prevent the firing of action potentials. In the right-hand panel the reciprocal connection is shown. Here the LSN is at resting potential with the GDN hyperpolarised to prevent firing of action potentials. Excitatory synaptic potentials can be seen in the GDN in response to the action potentials being produced by the LSN. These potentials disappeared when the LSN was hyperpolarised. Current injections are marked by arrowheads.

(b) Recordings showing a mixed electrical and chemical connection between a LSN and GDN. A burst of action potentials in the LSN in response to depolarising current injection gave rise to a two component response in the GDN. An initial increase in spike frequency occured before the inhibition from the chemical connection takes effect. Excitatory synaptic potentials from the electrical connection can be observed imposed on the chemically-mediated hyperpolarisation. These potentials were abolished when the prejunctional LSN was hyperpolarised. The LSN was at resting potential (-55 mV) and the GDN was hyperpolarised from its resting potential (-55 mV). Depolarising current injection into the LSN is indicated by the arrowhead.


LSN
-60 mV
20 mV
2 s
GDN
-55 mV
-50 mV
30 mV
2 s
20 mV
2 s
-60 mV

(a)


GDN
-55 mV
10 mV
2 s
LSN
-60 mV
10 mV
2 s

(b)

these connections formed and decayed over four days, with no connection between the neurones on days one and four. Chemical junctions were recorded on days two and three. The other example of this gradual decay occurred over three days, with chemical connections present on days one and two.

Only three pairs of the ten detected as having chemical junctions on day one survived to the second day. Of these one progressed to mixed and then electrical connections and the other two were eliminated. One of these junctions decayed over three days as described previously, the other was eliminated by day two of recording. Electrical connections between neurones were found not to decay in this manner. This type of connection, once formed, tended to persist for the duration of the pairs time in culture.

Neuronal pairs that were not connected on the first day of recording (n=18) often remained in this state on the second day (n=7). Three pairs that showed no connection on the first day of recording became electrical on the second day, one became chemical and one mixed. On one occasion a pair that showed no connection on days one, two and three of recording became chemically coupled on day four. Recordings from this pair of neurones over the four days are shown in figure 19. In five cases pairs that showed no connection on day one remained like this for three days in culture.

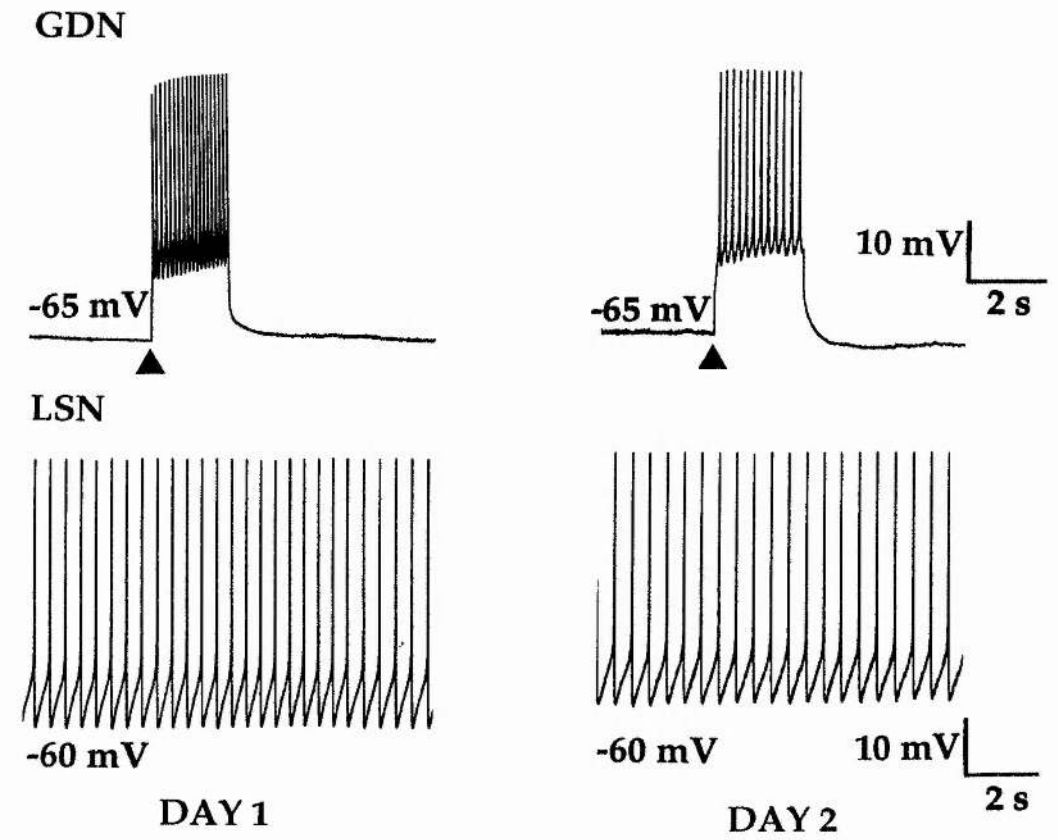
3.3.2 Neurite Extension.

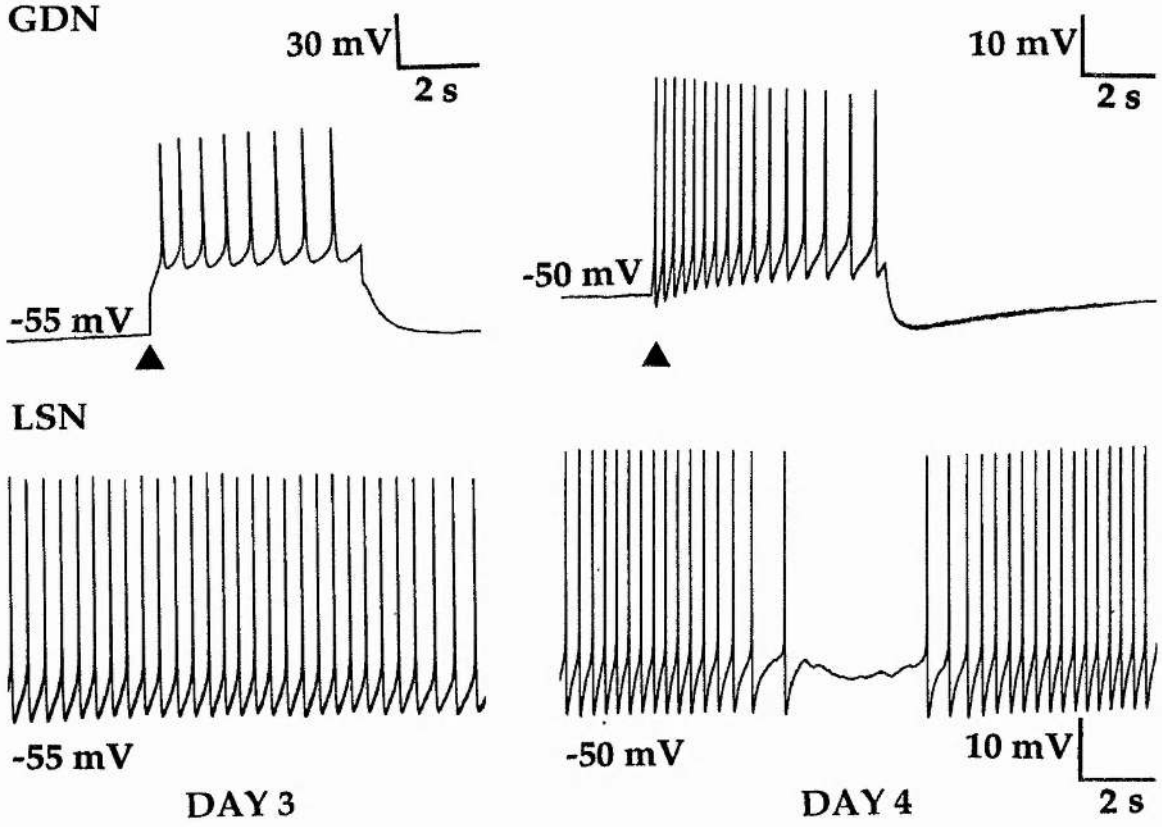
The pattern of neurite extension displayed by the GDN and LSN was slightly different to that observed for the B5 and B19 on 10 kDa MW substrate. Extensions by the B5 and B19 on this substrate tended to produce long thin neurites which showed fewer branches than GDNs and LSNs in identical growth conditions. The pedal neurones displayed more diffuse neuritic extension with a higher degree of branching. These neurites were mostly shorter and thicker than those produced by the buccal neurones. The neurites produced by B5 and B19 on 26 kDa MW poly-L-lysine more closely resembled those extended by GDN and LSN on 10 kDa MW substrate.

Little difference was observed between the growth cones of the

Figure 19.

Electrical recordings made from a GDN/LSN pair over a period of four days in culture conditions. The figure shows that on days one to three of recording no connection was present between the neurones in the direction shown. On the fourth day of recording a chemical connection was detected from the GDN to the LSN. In all four recordings the LSN resting membrane potential is shown. The GDN was hyperpolarised from resting membrane potential in the first two cases. On day one the resting potential was -55 mV and on day two it was -60 mV. The resting potential on days three and four was as shown on the recording. Depolarising current injections to the GDN are marked by the arrowheads.


GDN
-65 mV
10 mV
2 s
LSN
-60 mV
-60 mV
10 mV
2 s
DAY 1
DAY 2


GDN
30 mV
2 s
-55 mV
10 mV
2 s
-50 mV
LSN
-55 mV
-50 mV
10 mV
2 s
DAY 3
DAY 4

neurones in terms of appearance and size. GDN and LSN produced neurites which also displayed large numbers of varicosities along their length, as did the neurites of the buccal neurones.

3.3.3 Plating Neurones On Consecutive Days.

Experiments were carried out in order to establish if connections could be obtained between the giant dopamine neurone (GDN) and large serotonin neurone (LSN) if the neurones were plated on consecutive days. One of the neurones was placed into culture on day one and allowed to extend neurites overnight. The second neurone of the pair was then added on day two on top of the neurites extended by the first cell. The somata were often placed in contact and the axons were positioned so that they were overlapping. This plating regime allowed the area of contact between the two neurones to be maximised. Electrophysiological recordings were then made from the cell pairs on day three of the experiments. Photographs of two pairs plated in this way are shown in figure 20. In a few cases the neurone plated on the first day did not extend neurites to any great degree. The second neurone was always placed to allow contact between the two cell bodies and axons in such cases.

Some recordings from these experiments are shown in figure 21. From studying these electrophysiological recordings (n=13), both chemical (n=5) and electrical (n=3) connections could be detected. Connections were not detected in the remaining cases (n=5). Chemical connections formed from the LSN to the GDN on three occasions and in two cases from the GDN to the LSN. Chemical junctions formed when the LSN was plated first in three of the five cases. The remaining two chemical junctions were detected when the GDN was plated first. When the LSN was plated first, two connections were formed from the LSN to the GDN and one from the GDN to the LSN. Plating the GDN first gave one connection from LSN to GDN and one from GDN to LSN. The electrical connections were obtained when the LSN was plated first in two pairs and the other when the GDN was placed in culture first. All of the electrical connections were bidirectional. These results demonstrated that the technique could be used to obtain connections between neurones. Connections were able to form regardless of the amount of neuritic processes the participating neurones were producing.

Figure 20.

Upper plate. A GDN/LSN pair placed in culture on consecutive days. The GDN (top) was isolated on day one of the experiment and the LSN (bottom) was added on day two. The photograph shows the LSN on top of the neurites extended from the GDN. Electrical recordings from this pair of neurones revealed the chemical connection from the LSN to the GDN shown in figure 21 (a). Scale bar= 100 μm.

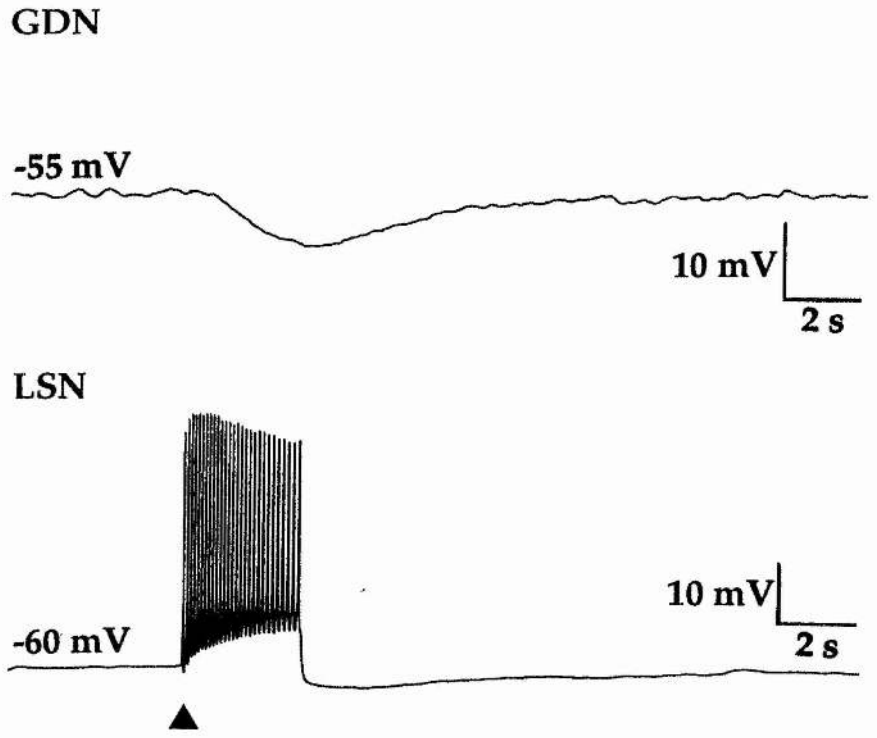
Lower plate. Another GDN/LSN pair used in consecutive day experiments. The LSN (left) was placed in culture on day one and the GDN (right) was added on day two. Recordings from this pair revealed the electrical connection shown in figure 21 (b). Scale bar= 100 μm.

Figure 21.

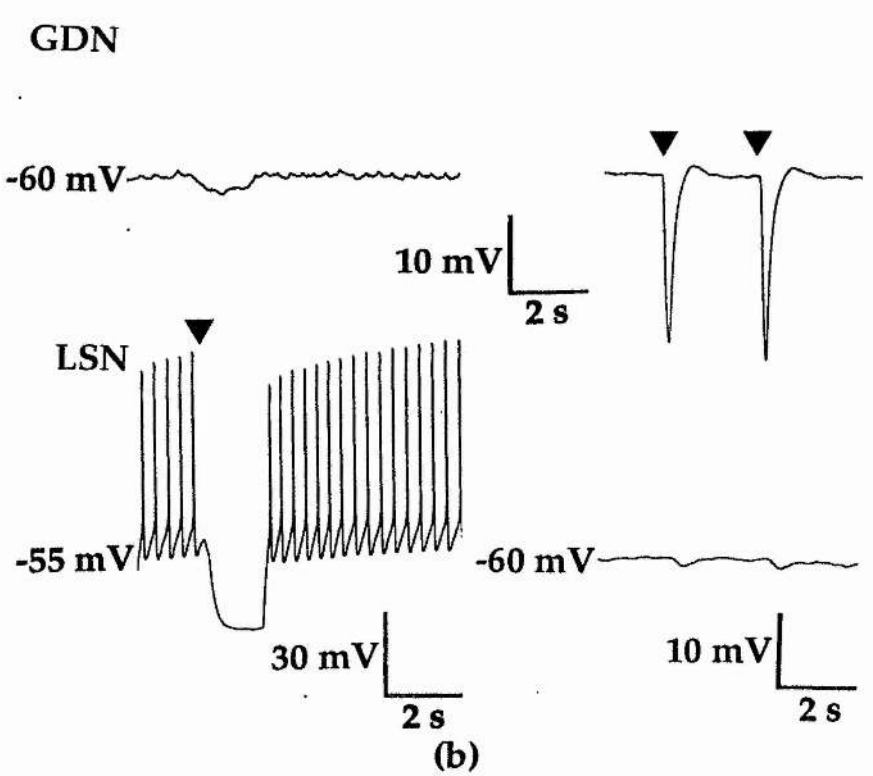
Electrical recordings from neurones placed in culture on consecutive days.

(a) This recording shows a chemical connection from a LSN to a GDN. The GDN was at resting membrane potential during this recording. Resting potential for the LSN was -55 mV. Depolarising current injection was made at the point marked by the arrowhead. The neurones that were involved in this connection are shown in the upper plate in figure 20.

(b) A reciprocal electrical connection recorded from the neurones shown in the lower plate in figure 20. In these recordings GDN was at resting potential. The LSN was at resting potential in the left-hand panel but was hyperpolarised to prevent the firing of action potentials in the right-hand panel. Hyperpolarising current injections were made at the points indicated by the arrowheads.


GDN
-55 mV
10 mV
2 s
LSN
-60 mV
10 mV
2 s

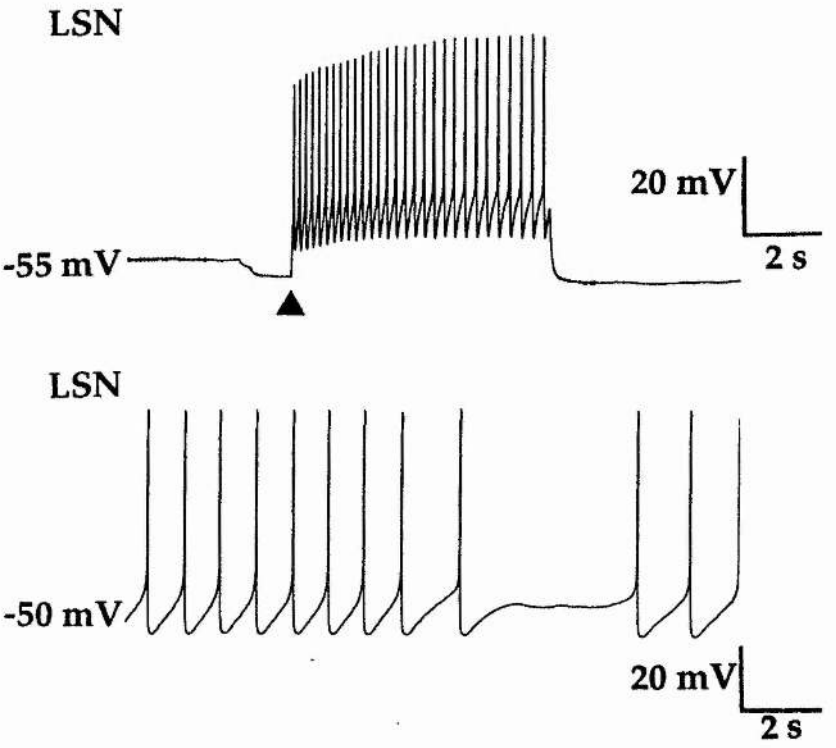
(a)


GDN
-60 mV
10 mV
2 s
LSN
-55 mV
30 mV
2 s
-60 mV
10 mV
2 s

(b)

Figure 22.

A weak chemical connection recorded between two LSNs. Both neurones were at resting membrane potential (shown on records). The record shows an initial slowing of spike frequency in the postjunctional neurone just before the main hyperpolarisation of the membrane. The point of depolarising current injection into the prejunctional LSN is marked by the arrowhead.


LSN
20 mV
-55 mV
2 s
LSN
-50 mV
20 mV
2 s

3.4 CIRCUIT EXPERIMENTS.

Having established that chemical connections could be obtained by plating the neurones on consecutive days, it was decided to use this method to construct small circuits of neurones. This was again to ensure the maximum area of contact between the two neurones in an attempt to increase the chances connections forming.

The LSN was chosen as the third neurone for these circuits, since chemical connections had been previously observed between two such neurones. These LSNs were not plated in a specific pair with each other or any GDNs, but had extended neurites which had overlapped. An example of a connection detected between two LSNs is shown in figure 22.

The circuits that were constructed in culture therefore consisted of two LSNs and one GDN. The procedure used for these experiments was as follows: one neurone was isolated on day one and allowed to extend neurites overnight. The remaining two neurones were added to the culture dish on day two. They were plated as close together as possible on the neurites put out by the first LSN, with axons and somata close together. Simultaneous recordings were then made form the three neurones on the third day of the experiment. Neurones were not plated on three successive days since by the third day the neurones were found to be unable to stick to the poly-L-lysine substrate unless left for several hours. This made such experiments impractical. An example of one of the circuits obtained (circuit three) is shown in figure 23. This photograph shows the large amount of contact that could be achieved between neurones when plated in this manner.

Only four circuits of ten that were constructed remained intact for recording. All of the neurones were found to be connecting with two partners via electrical or chemical junctions in three of the circuits recorded from. In the fourth case only one of the neurones had formed connections with the two other cells. The circuits are shown schematically in figure 24.

Connections between the neurones in these circuits were often difficult to detect due to their weak nature. This made studying these

Figure 23.

Photograph showing one of the three neurone circuits (circuit 3) consisting of one GDN (top) and two LSN (middle and left). The photograph shows the extensive overlap of the neurites that could be achieved using this plating regime. The electrical recordings made from these neurones are shown in figures 28, 29 and 30. Scale bar= 100 μm.

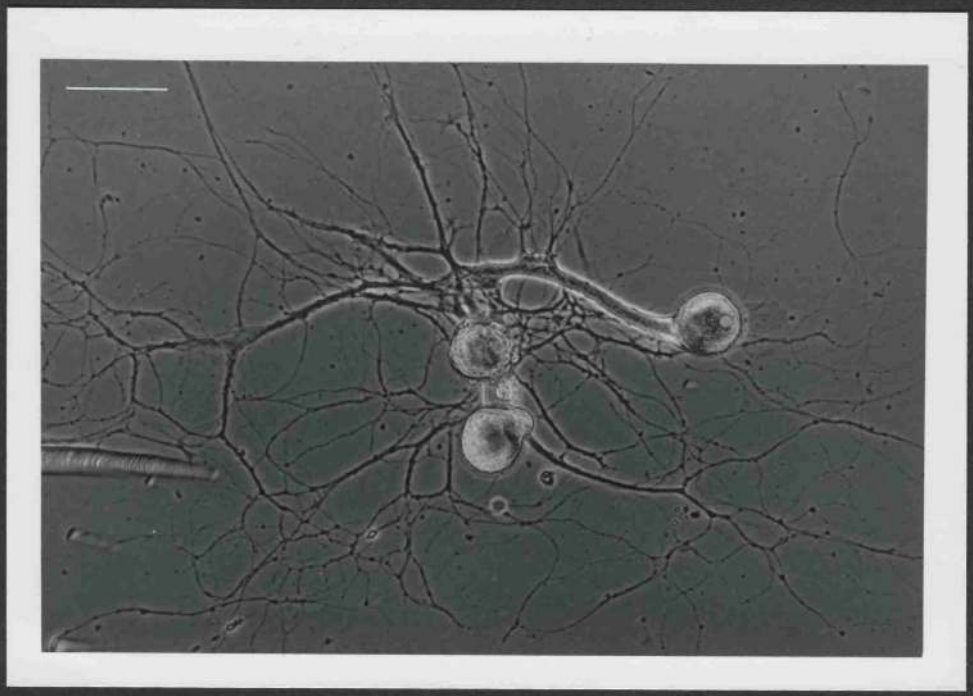
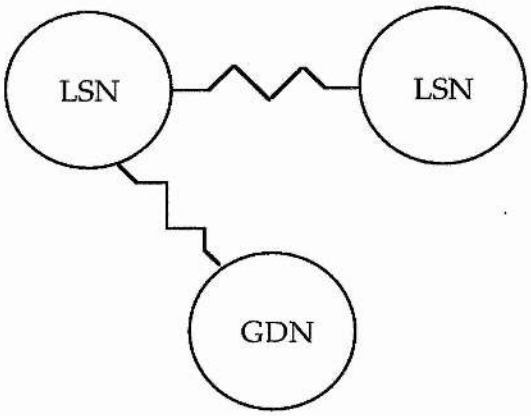
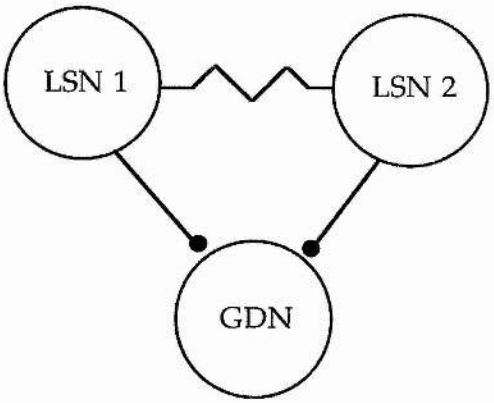


Figure 24.

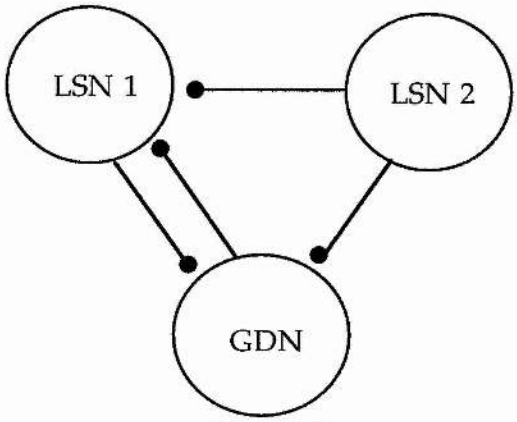
Schematic representation of the four circuits formed in cell culture. LSN= Large serotonin neurone. GDN= Giant dopamine neurone. LSNs marked 1 and 2 in circuits two and three correspond to those referred to in the text and figures 25 -30. Inhibitory chemical connections are denoted by filled circles and electrical connections by symbol —∿∿— .



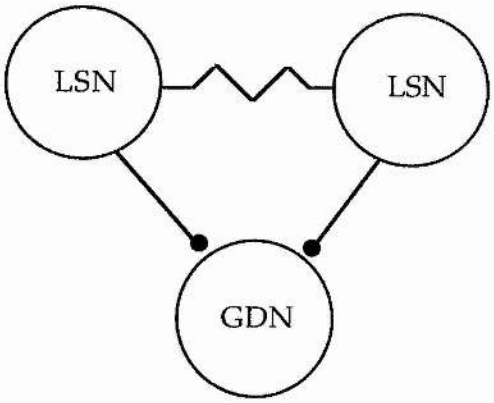
Circuit 1



Circuit 2



Circuit 3



Circuit 4

trios very difficult. From the four circuits that were formed, chemical (n=7) and electrical (n=4) connections were detected. Only one possible connection that could have been formed was not made. Connections between the individual pairs making up the circuits were studied by applying current to the neurones to keep them quiescent. One of the two neurones to be studied was then brought up to its threshold potential, allowing it to fire action potentials. The second neurone of the pair was then depolarised to fire a high frequency burst of action potentials to test for chemical connections, or hyperpolarised to test for electrical connections. All the pairs which made up the circuits were tested in this way.

A single reciprocal chemical connection was found with the rest being unidirectional (n=6), including one connection between two LSNs. The remaining five connections were all from LSN to the GDN. The other three LSN/LSN connections were electrical, with the remaining electrical junction being formed between a GDN and LSN. All of the electrical connections were reciprocal.

When the three neurones in the circuits were allowed to interact with each other, no effects could be detected between the neurones in two of the four trios. Circuit one had only two weak electrical connections present, and circuit four had extremely weak connections between the three neurones. In the remaining two cases, where connections were stronger, some circuit interactions could be observed. The circuit interactions were studied by firstly returning all the neurones to their resting membrane potentials. If any of the neurones were quiescent at this potential they were depolarised to their threshold potential. Each of the three neurones were then depolarised, in turn, to fire high frequency bursts of action potentials in order to see the effect on the overall output of the circuit.

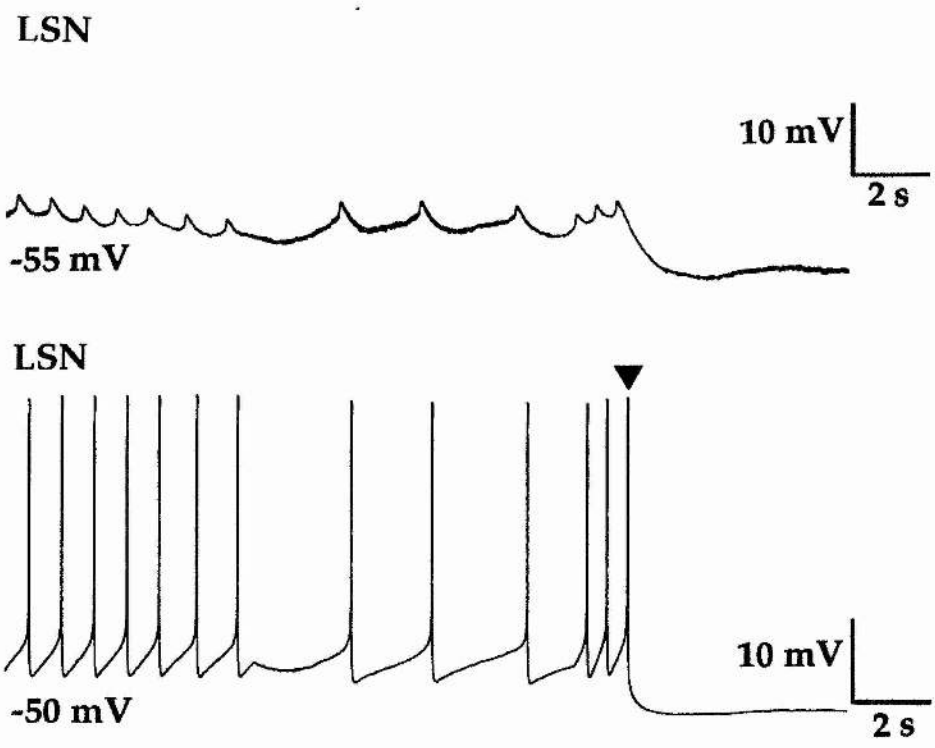
In circuit two the chemically mediated inhibition of the GDN by one of the LSNs was observed, but only after strong depolarisation of the LSN involved. The weak electrical coupling between the two LSNs was also present in this trio, but since the coupling was weak its effect on the overall activity of the circuit was difficult to deduce. The individual connections from this circuit are shown in figures 25 and 26 and the circuit interactions in figure 27.

Figure 25.

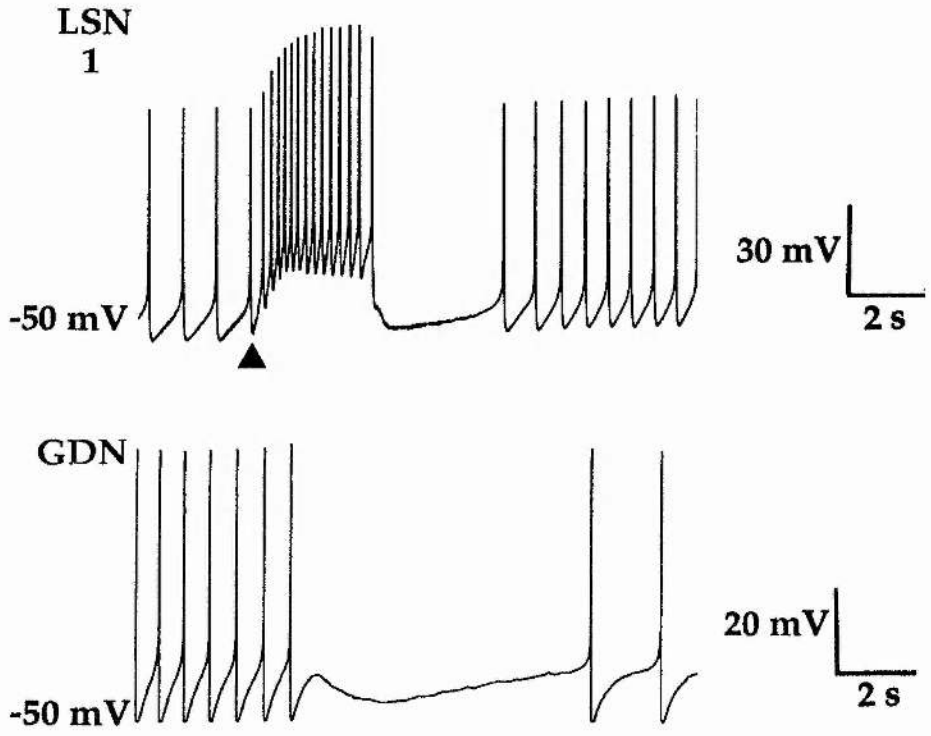
Electrical recordings from individual neurone pairs from circuit 2.

(a) The weak electrical connection formed between the two LSN neurones of this circuit. Both neurones were at resting potential which is shown on the records. Hyperpolarising current was injected into LSN at the point marked by the arrowhead.

(b) The chemical connection detected between LSN 1 and the GDN. These neurones were recorded from at their resting membrane potentials which are shown on the records.


LSN
10 mV
2 s
-55 mV
LSN
10 mV
2 s
-50 mV

(a)


LSN
1
30 mV
2 s
-50 mV
GDN
20 mV
2 s
-50 mV

(b)

Figure 26.

This figure shows the remaining individual neurone pair recording from circuit 2. This was the chemical connection from LSN 2 to the GDN. The GDN was recorded from at its resting potential which is shown on the record. Hyperpolarising current was injected into LSN 2 removing it from its resting potential of -55 mV. The arrowhead marks the point where depolarising current was injected into the LSN.

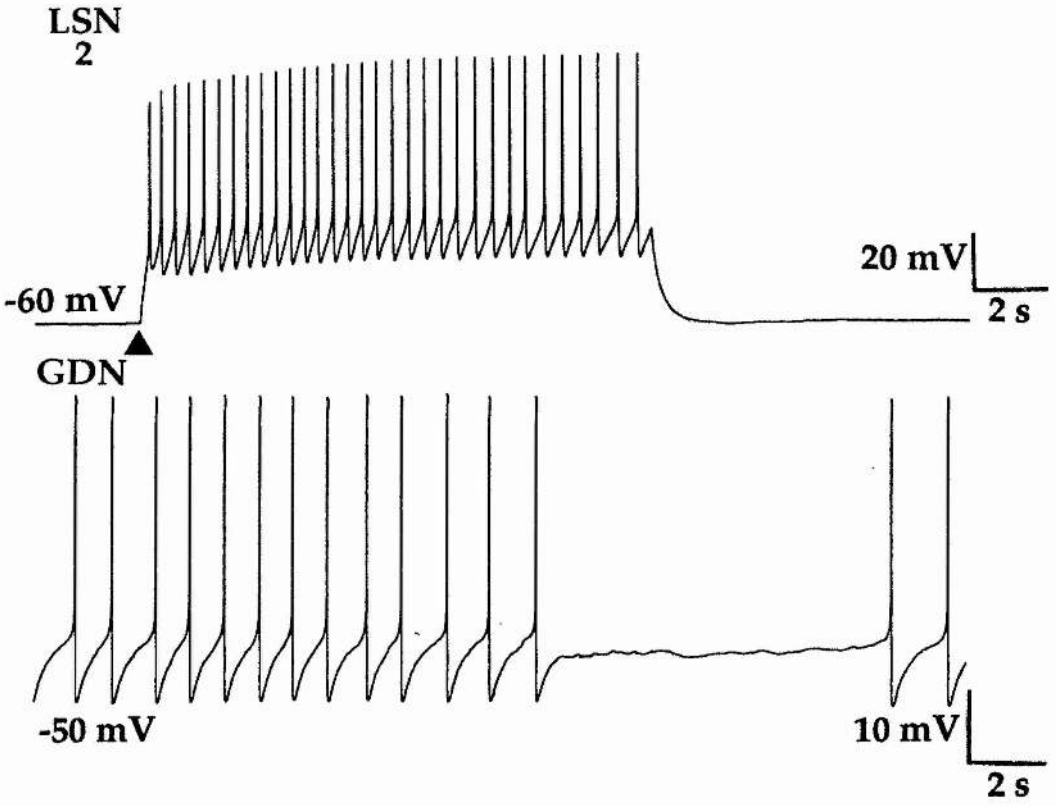
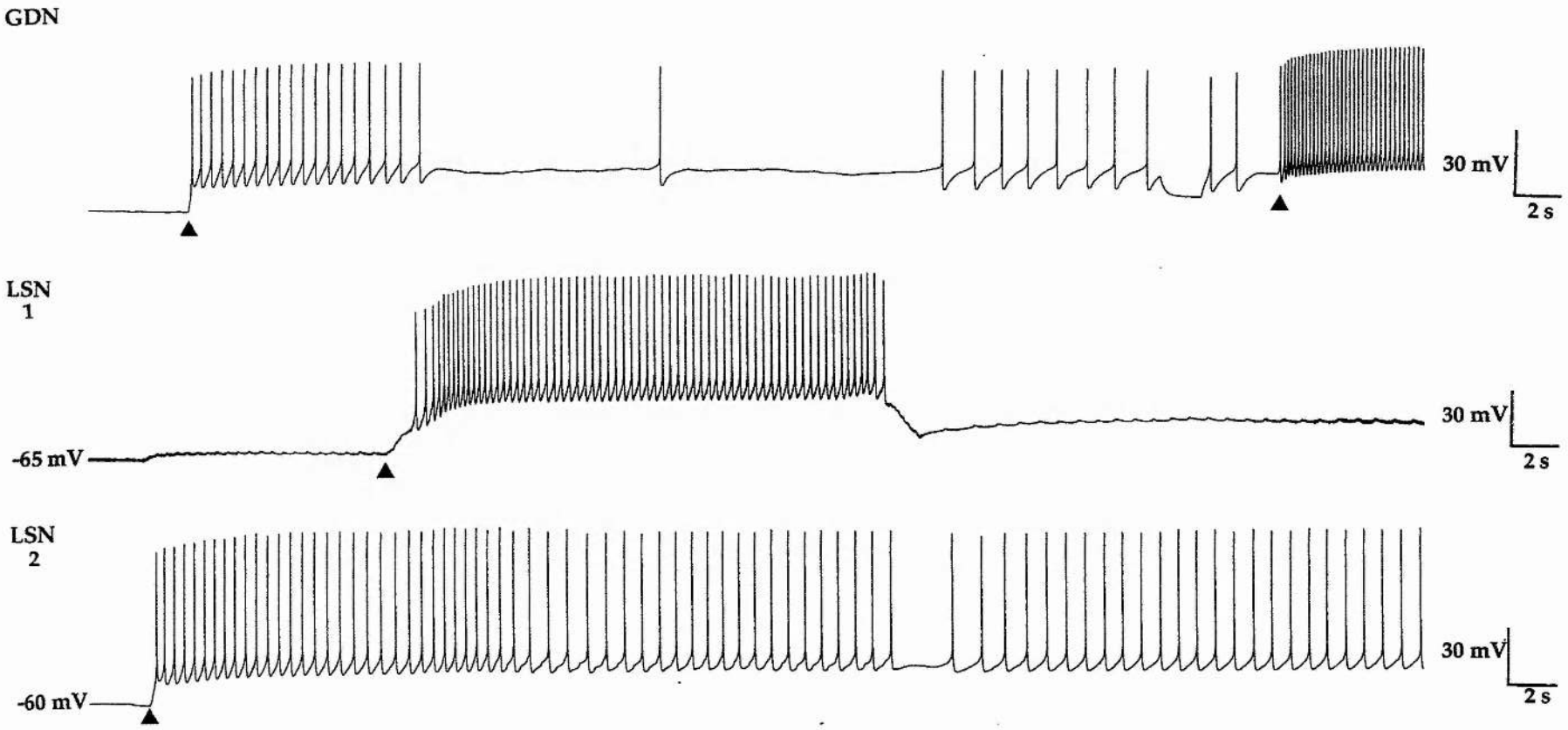

LSN
2
-60 mV
20 mV
2 s
GDN
-50 mV
10 mV
2 s

Figure 27.

The overall activity of circuit 2 when all three component neurones were firing at the same time. The circuit interactions were studied by returning two neurones to their resting potentials and injecting depolarising current into the third to produce a high frequency burst of action potentials. In the record shown LSN 1 was depolarised to produce a high frequency burst to observe its effects on the GDN (-50 mV resting potential) and LSN 2 (-55 mV resting potential). LSN 1 caused inhibition of the GDN, totally abolishing firing activity in this neurone. The weak electrical connection between the two LSNs was also apparent from LSN 1 to LSN 2 during the period when LSN 1 was being depolarised and when the activity in this neurone was stopped. When similar tests were carried out with the other two neurones no effects could be observed. Depolarising current injections were made at the points marked by the arrowheads.


GDN
30 mV
2 s
LSN
1
-65 mV
30 mV
2 s
LSN
2
-60 mV
30 mV
2 s

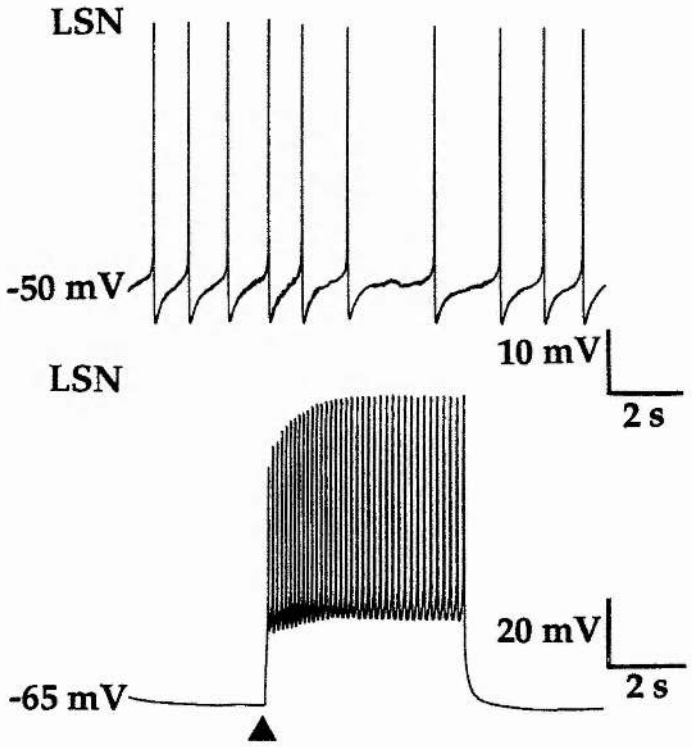
One of the LSNs in the circuit three was inhibited by the GDN, but as with circuit two, only after strong depolarisation of the GDN. A weak interaction between the two LSNs was also present in this trio. The second LSN weakly inhibited the first when both neurones were spontaneously active. The connections detected between the individual pairs from circuit three are shown in figures 28 and 29. Recordings of the three neurones interacting with each other are shown in figure 30.

Figure 28.

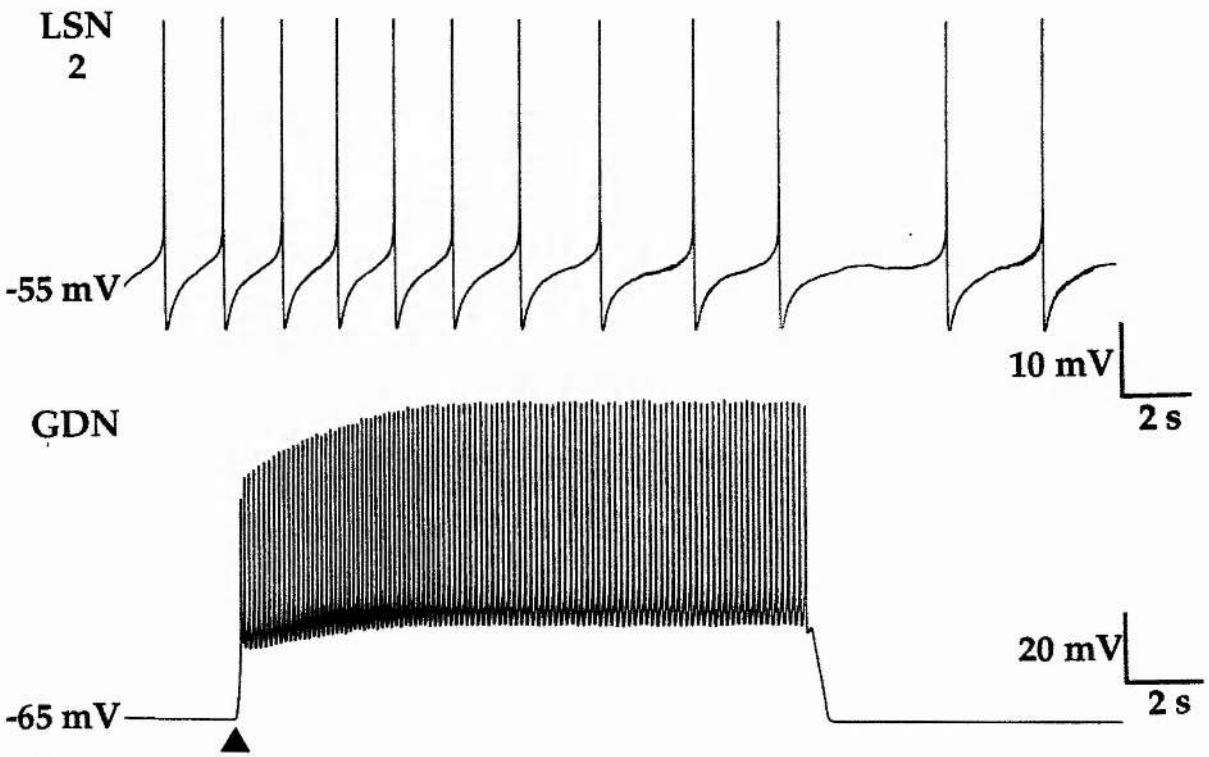
Electrical recordings made from individual pairs from circuit 3.

(a) The weak chemical connection detected between the two LSNs from circuit 3. The LSN on the upper trace was at resting potential and the second LSN was hyperpolarised from its resting potential of -55 mV.

(b) A weak chemical connection between the GDN and LSN 2. The LSN was at its resting potential and the GDN was hyperpolarised from its resting potential of -55 mV. The arrowheads mark the point of depolarising current injection to the prejunctional neurone.


LSN
-50 mV
10 mV
2 s
LSN
20 mV
2 s
-65 mV

(a)


LSN
2
-55 mV
10 mV
2 s
GDN
20 mV
2 s
-65 mV

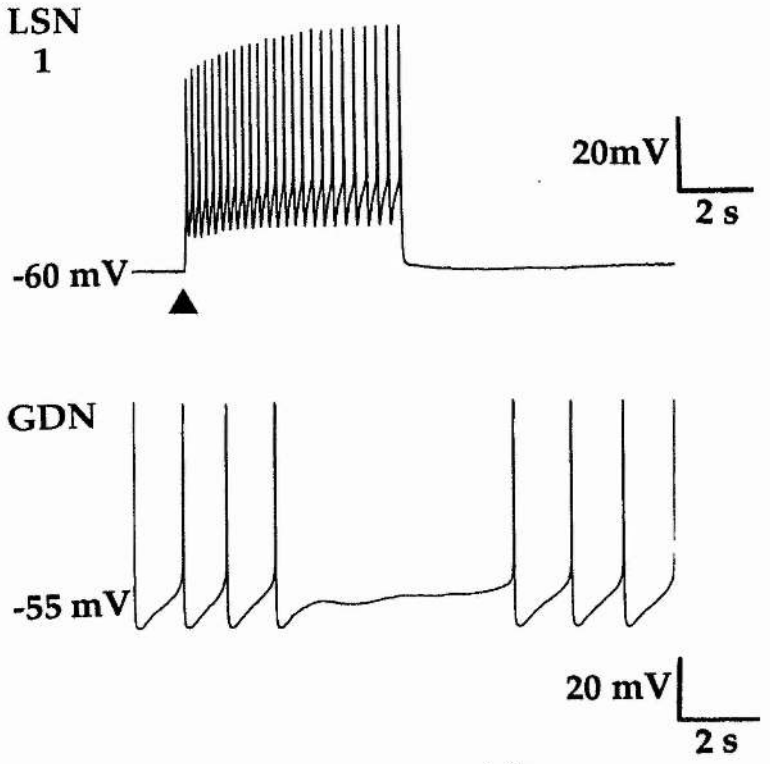
(b)

Figure 29.

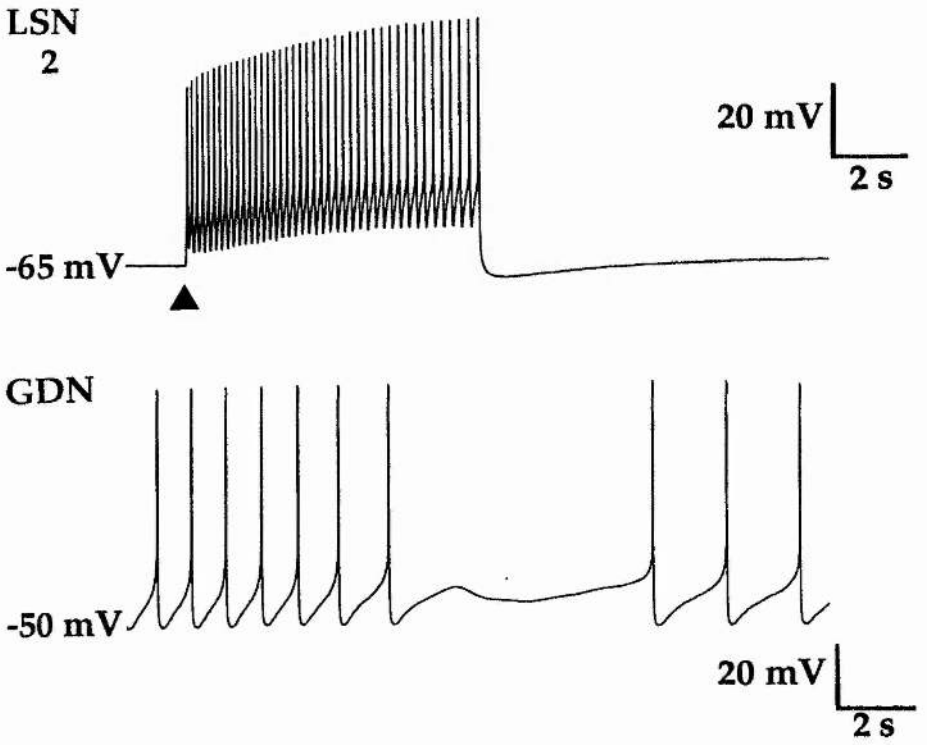
Electrical recordings made from individual neurone pairs of circuit 3.

(a) Recording of the chemical connection detected between LSN 1 and the GDN. The GDN was at resting potential (shown on record). The LSN was hyperpolarised from its resting potential of -50 mV.

(b) The chemical connection detected between LSN 2 and the GDN. Again the LSN was hyperpolarised from its resting potential of -55 mV. The GDN was slightly depolarised from its resting potential of -55 mV to allow it to fire action potentials. Depolarising current injections were made into the prejunctional neurone at the points marked by the arrowheads.


LSN
1
20mV
2 s
-60 mV
GDN
-55 mV
20 mV
2 s

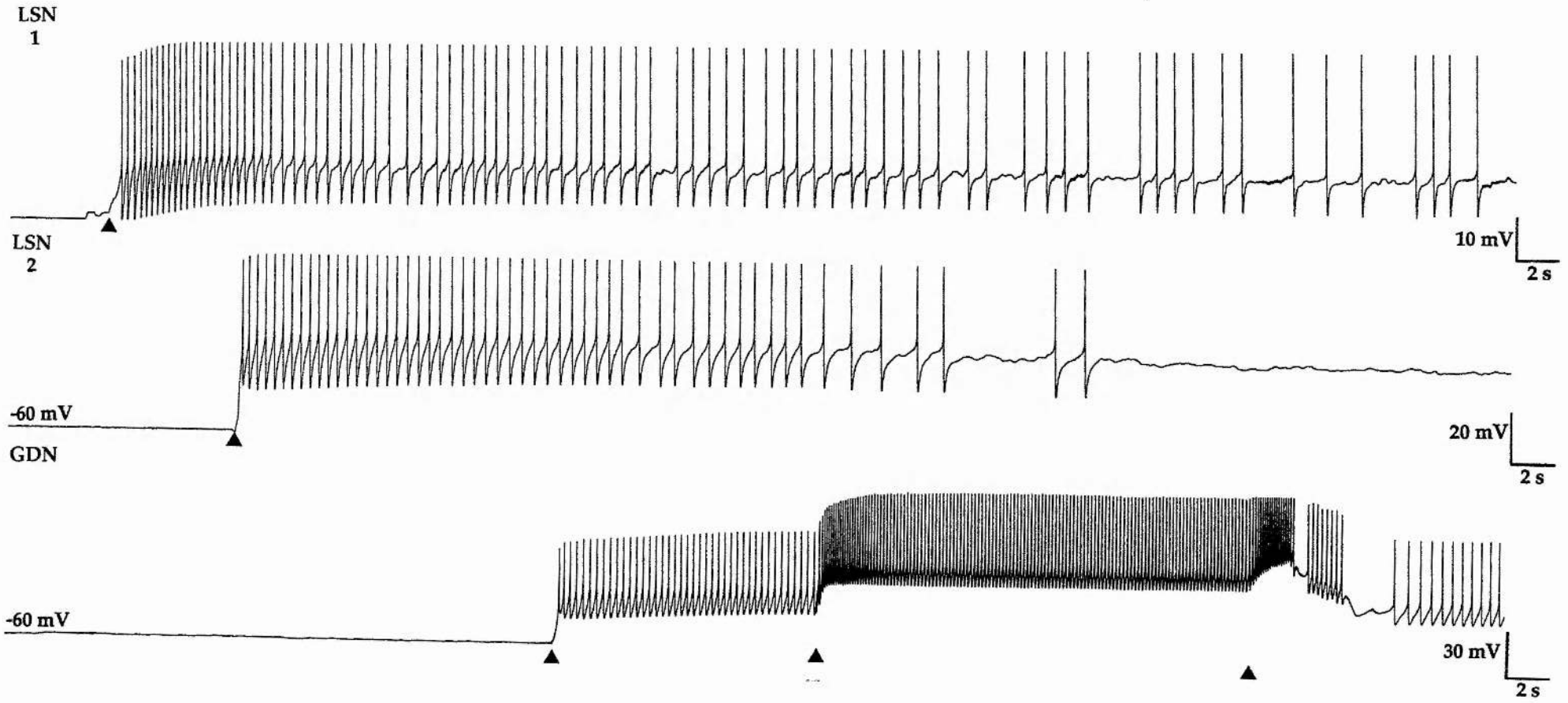
(a)


LSN
2
20 mV
2 s
-65 mV
GDN
-50 mV
20 mV
2 s

(b)

Figure 30.

This figure shows the electrical recordings made from the three neurones of circuit 3 when they were all active. The initial depolarisation on each record returned LSN 2 and the GDN to their resting potentials. LSN 1 was depolarised slightly from its resting potential of -50 mV so that it was above its threshold potential for firing action potentials. Circuit interactions between the neurones were studied by increasing the firing frequency of one of the neurones and observing the effects on the other two neurones. In the record shown LSN 2 was inhibited weakly by the GDN when GDN was initially at its resting potential. Increasing the firing frequency of the GDN lead to a more marked inhibition of LSN 2. A weak inhibition of LSN 1 by LSN 2 was also seen when both neurones were active. When this effect was further investigated by increasing the firing frequency of LSN 2 no inhibition was observed (not shown). These were the only interactions observed between these neurones. The points at which injections of depolarising current were made into the three neurones are indicated by the arrowheads.


LSN
1
LSN
2
10 mV
2 s
-60 mV
GDN
20 mV
2 s
-60 mV
30 mV
2 s

CHAPTER 4
DISCUSSION

This study was carried out in an attempt to construct a small circuit of neurones and study their interactions. In order to do this, neurones had to be chosen which would form connections with each other in cell culture. The B5 and B19 neurones from the *Helisoma* buccal ganglia were initially chosen since they had been reported as forming a chemical connection *in vitro* (Haydon, 1988). These neurones were replaced by the giant dopamine neurone (GDN) and large serotonin neurone (LSN) from the left and right pedal ganglia, respectively. The GDN and LSN had also been shown to form chemical connections in culture (Stuart Harris, unpublished observations). Three neurone circuits were eventually constructed but experimentation on them was found to be difficult. The reasons for these difficulties, and other aspects of the project, will be discussed below.

4.1 EXPERIMENTS WITH B5 AND B19 NEURONES.

The B5 and B19 neurones used in the experiments reported in this study were chosen on the basis of work carried out previously (Haydon, 1988). This pair of neurones do not form a connection with each other *in vivo*. The reason that these neurones were picked for studying synapse formation is therefore unclear. The choice of buccal neurones was probably made since the buccal ganglia of *Helisoma* has been well studied in the past (Kater, 1974), and the B5 and B19 are the most prominent pair of neurones in these ganglia for dissection. This has lead to these two neurones being used in studies into the action of serotonin on growth cones, and in one such study the B5 and B19 were reported as forming electrical connections with each other (Haydon *et al*, 1987). The neurones were therefore probably picked for synapse studies on the basis of their ability to form electrical connections with each other.

The results obtained in this study differ from those reported previously for the B5/B19 pair (Haydon, 1988). In the previous study cholinergic chemical connections were detected from the B5 to the B19 after a period of two to four days in culture conditions. These neurone pairs were in contact via their soma and had no axonal or neurite projections. The results presented in this study report that the B5 and B19 were seen only to make electrical connections with each other.

Differences in methodology may account for the differences in the

results. Haydon removed the neurones from the ganglia and placed them into a medium containing 50% haemolymph where the neurones were allowed to adhere to each other. The pairs were then placed into a medium containing 1% haemolymph until they were required for electrophysiological recording, at which time they were transfered to a culture dish containing L-15 medium. In these conditions the neurone pairs then firmly adhered to the poly-L-lysine substrate coating this dish (Haydon, 1988). Neurones in the present study were removed from the ganglia and placed directly into the recording dish, which contained L-15 medium, where they remained for the duration of the culture. This meant that the haemolymph treatment was not used. Haemolymph was also used by Haydon's group to treat neurone pairs which were in contact via neurites (Zoran *et al,* 1990).

Another difference was that Haydon removed the neurones complete with their axons, which were subsequently retracted whilst the neurones were in the 50% haemolymph medium. The experiments reported here with the B5 and B19 were carried out early in the project when the dissecting technique was still being mastered. This meant that practically all the buccal neurones were removed without any or with only a very small length of axon attached. The lack of the initial axonal segment may also help account for the differences in the connections formed by the neurone pairs, both in soma-soma contacts and those contacting via neurites. In the latter case the presence of this axon segment has been shown to be important for neurone survival and neurite growth (Schacher and Proshansky, 1983).

The B5 and B19 neurones are not connected to each other *in vivo* , meaning that any connection formed between these neurones in culture is a novel connection. This may explain the absence of any chemical connection between these neurones from a purely developmental stand-point. Inappropriate connections made within the nervous system are eliminated during the initial stages of neural development. This synaptic elimination may be expressed by the isolated neurones, since it may be imagined that these neurones would have to return to a developmental-like state in order to produce neurites and make contacts. Neurones in this situation may therefore also express the selectivity of connection formation observed in developing nervous systems. However the results obtained by Haydon's group would suggest that this

possibility is unlikely.

A developing neurone requires interaction with various chemical signals during the period in which it is growing and forming connections. If such environmental stimuli are not present the neurones may conceivably not develop in the correct manner, leaving them unable to form connections. This is a possible explanation for the lack of chemical connections detected in B5/B19 pairs placed in culture conditions consisting of defined medium alone. It may also explain the success that Haydon had in pretreating neurones with haemolymph before recording. Haemolymph has been shown to aid the survival of neurones in cell culture (Schacher and Proshansky, 1983), and it may also provide some factor which promotes the formation of synapses.

Placing the B5/B19 pairs into conditioned medium did not appear to have any effect on the formation of chemical connections between them. This suggests that either environmental cues contained within the conditioned medium are not sufficient to aid in chemical connection formation, or that lack of some chemical stimuli was not the reason that these neurones failed to form chemical connections. Haemolymph was not added to the conditioned medium as previously reported (Zoran *et al,* 1990). The medium may therefore have been lacking in some signal from the haemolymph which the developing neurone required for synaptogenesis.

The B5/B19 neurones were placed in close apposition, particularly those in the soma-soma contacts. It may be expected therefore that some effect of general release of neurotransmitter, from the soma and neurites of one neurone, and general detection, by receptors over the surface of the second neurone, may occur. This did not appear to happen however, suggesting that this type of detection does not occur. Further work by Haydon (Haydon and Zoran, 1989) does however indicate that this type of release and detection is capable of taking place. In this work the B19 neurone was used as an assay neurone for the release of acetylcholine from the B5 and showed that by manoeuvering the B19 close to the B5 transmitter release could be detected. This may suggest that release of neurotransmitter was not occurring from the neuronal somata in the soma-soma experiments carried out in this study.

Release of neurotransmitter from the B19 neurone would not have been expected as it has previously been shown that this neurone does not begin to release transmitter until it has had prolonged contact its appropriate target (Zoran *et al,* 1990). The B5 neurone should however be capable of releasing transmitter after a few seconds of contact with the B19 (Haydon and Zoran, 1989). These observations suggest that the neuronal somata in the soma-soma experiments, and the neurites in experiments involving extension were unable to release any neurotransmitter, due possibly to a lack of release sites on this area of the neurone or a lesion in the spike-transmitter release coupling mechanism. The latter of these could have involved some kind of breakdown in the link between calcium ion entry and vesicle mobilisation and fusion to the presynaptic membrane. A lack of appropriate voltage-sensitive calcium channels in the region of the contact between the neurones would prevent the spike-release coupling mechanism from functioning at all. Unless the lack of calcium channels was explained by non-expression of the channel proteins, this possibility does not seem likely in light of previous work on isolated leech neurones (Ross *et al,* 1988). Other presynaptic mechanisms may also have been responsible. For example the synthesis of neurotransmitter in the newly isolated neurone may have been impaired, leading to a lack of neurotransmitter in the release vesicles. A final possibility that could have prevented transmitter release is a fault in the fusion of the neurotransmitter-containing vesicle to the presynaptic membrane.

Alternatively there may have been release of transmitter from the B5 neurone, meaning that the lack of chemical connections was due to a fault in the postsynaptic machinery. The simplist explaination for a breakdown in communication on the postsynaptic side would be a lack of appropriate receptors present on the B19 somata to detect any released acetylcholine from the B5. Alternatively the receptors may have been present on the B19 but the coupling between them and the chloride ion channels could have been impaired. This coupling was dysfunctional activation of the acetylcholine receptor on the B19 would not lead to opening of the ion channel, and therefore a response would not be observed in the postjunctional neurone.

When Haydon's group isolated B5 and B19 neurones from the buccal ganglia they removed sections of the axons attached to the neuronal

somata. These axons were then encouraged to retract by placing the neurones in haemolymph for the soma-soma experiments (Haydon, 1988), or were left to aid in the production of neurites if so required (e.g. Haydon and Zoran, 1989). It is possible that by encouraging the axons to retract this enables the neurones to express any axonal release sites or receptors on their somata. This is a strong possibility since most molluscan synapses are between the axons of the two participating neurones. In cases where no or very little axon was initially present this possibility would not have been open, and this could have contributed to the lack of chemical connections between B5 and B19 neurones involved in soma-soma contacts.

The lack of chemical connections between B5 and B19 neurones which were extending neurites could also have been due to a lack of the appropriate release sites and receptors. This may have occured because of the lack of an axon on these neurites when they were first isolated. The presence of the initial axon segment has been shown to be important in the formation of chemical connections (Nicholls *et al,* 1990; Hawver and Schacher, 1993). Alternatively connections could have been present amongst the neurite branches and were not recorded at the soma because of the distance between the site of contact and the point of recording. This seems unlikely since small electrical synaptic potentials were often detected, as were what appeared to be chemical excitatory synaptic potentials (see figure 10 b). The ability of these events to be seen at the point of recording in the cell body suggests attenuation of responses was not responsible for the lack of chemical connections detected.

The possible presence of excitatory synaptic potentials, which were detected in an unconnected pair during a period when neither neurone was active, may suggest spontaneous transmitter release can occur in these neurones. These occurrences indicate that both receptors and release sites may be present on the neurites extended by these neurones. The presence of these potentials on the B19 neurone points towards this neurone detecting spontaneously released transmitter from the B5, or possibly from the B19 itself. Of these two possibilities the first is more likely, not least because of the previous study which showed that B19 only begins to release transmitter after contact with its target muscle (Zoran *et al,* 1990).

It has been suggested that the interactions between the neurites of growing neurones are dependent upon the presence of specific antigens on the membranes of the neurones in question. If the B5 and B19 neurones do not express the appropriate cell surface antigens then chemical synaptogenesis between the neurones would be suppressed.

The pattern of neurite extension from the buccal neurones in isolation tended to be multipolar. This type of extension from numerous points of the neuronal cell membrane tends to occur when a neurone is isolated without its axon attached and is similar to the situation seen in many vertebrate neurone cultures (Craig and Banker, 1994). In these vertebrate system the neurones tend to develop neurites from an initially round cell body, due to the isolation technique. From this starting point neurites grow out from many points around the membrane giving the neurone a multipolar appearance. In some cases one of the extending neurites is selected by the neurone as the axon, and develops as such. This process may have taken place in the multipolar buccal neurones if they had been observed over a longer period of time. As stated earlier most synapses in molluscan nervous systems are axo-axonic, and the development of axons in this way may have allowed the formation of connections between the neurones.

Although no chemical connections formed between B5 and B19 neurones, electrical connections were detected between neurones involved in soma-soma contacts and those interacting via neurites. The presence of gap junctions between these neurones may be another indication of the de-differentiation of the adult neurone back into a developmental state. Gap junctions are known to be important in the development of many systems (Caveney, 1985) and it is possible that the formation of gap junctions between these isolated neurones is an expression of this. Novel electrical connections have previously been reported between buccal neurones, including the B5 and B19, *in situ* (Bulloch and Kater, 1981; Hadley *et al*, 1983). Therefore the electrical connections recorded *in vitro* between these neurones are not unprecedented.

The formation of electrical connections between neurones interacting via neurites suggests that the neurones are capable of transporting intracellular material along the neurites. This means that the lack of

chemical connections was unlikely to be due to an inability to transport the structures required for release sites and receptor sites along the newly extended neurites. Electrical connections between B5 and B19 neurones have previously been shown to require both neurones to be actively extending neurites (Haydon *et al,* 1987), and from the results obtained here this would seem to be the case. In this study the investigators also show that neurone pairs in which one partner has had its neurite extension stopped, do not form any type of connection. These findings suggest that the B5 and B19 pair of neurones are unlikely to form a chemical connection whether the neurones are extending neurites synchronously or not.

4.2 EXPERIMENTS WITH THE GIANT DOPAMINE NEURONE (GDN) AND LARGE SEROTONIN NEURONE (LSN).

The chemical connections recorded between pairs of giant dopamine neurones (GDN) and large serotonin neurones (LSN) were similar in nature to those obtained by other workers (Syed *et al,* 1993). One main difference was that reciprocal chemical connections were not recorded between the neurones in the present study, whereas Syed detected such connections in the majority of his experiments. A reciprocal connection is present between the neurones *in vivo* (Syed *et al,* 1993). The reason why no such connection was re-established under culture conditions is unknown.

The GDN/LSN pairing formed inhibitory chemical connections which had slow latency and a long duration. These connections were only seen in response to a high frequency burst of action potentials in the prejunctional neurone. One-to-one responses to individual action potentials were not seen. This observation brings into question whether these junctions are chemical synapses or merely detection by one neurone of neurotransmitter released from all over the neuronal processes and soma of the other neurone. The connections were possibly more indicative of such general detection rather than the more subtle process that would occur at a synapse, where pre- and postjunctional machinery are in close apposition. However, if general detection was to explain the connections observed between the GDN/LSN pairs, then pairs may have been expected to be involved in reciprocal chemical

connections, rather than unidirectional connections. It may also have been expected that reciprocal chemical connections should have been recorded between every pair tested if general release and detection of transmitter was taking place. The small amount of neurotransmitter that would be released during prejunctional activity would also tend to go against the idea of general release and detection. This is because of the distance that released transmitter may have to travel before detection by receptors could take place. The chemical connections recorded are therefore unlikely to be accounted for in this way.

Rather than a situation where there may be a fairly large distance between the prejunctional release site and the postjunctional receptors, the connections recorded may have been from receptors close to the release site. These areas may not be as closely associated as may be expected at synapses, but the receptors and transmitter release sites may be close enough to allow the small quantities of transmitter released to be detected postjunctionally following a short period of diffusion. This type of association between the pre- and postjunctional structures could go some way to explaining the lack of any one-to-one correspondence between prejunctional action potentials and postjunctional responses.

If synapses were present between neurones, individual synaptic potentials may have been present at the site of the junction and not recorded at the cell body due to effects of the space constant. This would mean that a response may be present at a point or points in the neuritic processes of the postjunctional neurone, but may have been attenuated as it was communicated back to the point of recording in the soma. Therefore the original potential at the point of the junction may be totally lost by the time it has travelled to the recording electrode. This may explain why only large compound responses were obtained to bursts of action potentials in the prejunctional neurone. This explanation of the apparent lack of one-to-one responses would only be valid if all of the connections obtained were present on long neurites produced by the isolated neurones. In many cases however the neurones were contacting via the initial axon segment (see figures 14 and 15). Individual synaptic potentials were still not detected between such pairs of neurones. This suggests either that even short distances may have been enough to completely attenuate the individual responses, or that connections were not being made in these regions.

A problem with the space constant explanation occurs with the electrotonic potentials which were often recorded (see figure 18). In these cases action potentials in one neurone are communicated to its partner, where they give rise to a subthreshold electrotonic response. The communication of this electrotonic potential to the soma of the neurone will abide by the same laws as the chemically induced potential, meaning that the size will be reduced with increasing distance from their point of origin. These electrotonic potentials were very small when they were detected (1-2 mV) and taking into account that the neurones with electrical connections often had diffusely branched neurites, similar to those shown in figure 14, then the original response may have been rather large in nature. Alternatively electrical junctions may have formed preferentially at sites close to the soma, and were therefore easier to detect.

The appearance of mixed chemical and electrical connections adds to the debate about the role of the space constant. In these pairs the small electrotonic potentials were easily recorded (see figure 18 b). But as with other chemically connected pairs, individual one-to-one chemical responses were not recorded. This means that if the space constant were to explain the lack of chemically mediated individual potentials then the electrotonic responses must have been larger than the chemically induced event.

Probably the best explaination of why no individual synaptic potentials were seen relates to the slow characteristics of the responses. This means that individually generated inhibitory potentials would merge to give the appearance of a smooth hyperpolarising potential.

Electrical connections were often recorded between GDN/LSN pairs after one day of isolation. These connections persisted for the duration of the pairs time in culture. The formation of these novel connections between the GDN/LSN pairs is something of a mystery. The formation of these gap junctions between the neurones may be an example of de-differentiation in these neurones, leading to the expression of gap junctions which are important in development of many tissues (Caveney, 1985). The development of electrical connections between neurones is also often seen as a transient stage in the formation of a

chemical junction. For example, the neurones may use the gap junctions to align their membranes for the formation of synaptic sites, or for the transfer of some signalling molecules to initiate synapse formation. The data obtained in this study however suggests the opposite situation, where pairs form chemical connections early in the culture but become electrically coupled as the time in culture increases. Why a pair of neurones which are normally chemically coupled *in vivo* should reform an appropriate connection and then eliminate it is unknown. A possibility is that the culture conditions are responsible in some way, either from a lack of a nutrient or a necessary brain-derived signal molecule. Whatever the cause of the elimination it appears to occur in a gradual manner, usually taking two to three days for the change from chemical to electrical or chemical to no connection. This gradual process may suggest that the reason is metabolic or even genetic in origin.

When chemical connections were detected it was noted that they were three times more likely to be from the LSN to the GDN than vice versa. This observation gives rise to another mystery, since both neurones were plated at the same time. This means that any difference in the likelihood of one or other of the neurones becoming prejunctional is not related to the time that the neurones have been in the culture conditions. The reason for the difference may be a developmental property of the LSN which could allow this neurone to begin releasing neurotransmitter at an earlier time than the GDN. This could tie in with the known developmental importance of serotonin in the nervous system, particularly its ability to control neurite extension via effects on the neuronal growth cone (e.g. Haydon *et al,* 1984 and 1987).

Developmentally serotonergic neurones are amongst the earliest to appear in the nervous system of *Helisoma* (Goldberg and Kater, 1989) and also in mammals (Lauder *et al,* 1982). In both of these systems serotonin has been noted to have profound effects on the development of other neurones. This could mean that serotonergic neurones may develop slightly ahead of other neurones to provide the necessary cues for normal development. The LSN may therefore develop release sites at an earlier time than the GDN, and thus be able to act as a prejunctional neurone before the GDN. Indeed serotonin release has recently been reported from the growth cones of developing neurones from the neonatal rat brain (Ivgy-May *et al,* 1994), suggesting that growth cones

have all the characteristics of synaptic terminals. The problem here is that, in mammals at least, although serotonergic neurones are first to develop they are amongst the last to form connections (Lauder *et al,* 1982).

No differences could be seen in the rates of neurite elongation between the GDN and LSN in culture. Therefore any difference in the likelihood of LSN or GDN being the prejunctional neurone is unlikely to be down to a difference in neurite elongation. The reason that the LSN appears to be dominant in deciding the prejunctional neurone seems more likely to be developmental, for which the case is quite strong, or biochemical.

Another possibility is that the GDN to LSN connection does form transiently at the same time as the LSN to GDN connection. The GDN may then stop producing or releasing transmitter early in the culture, leaving only the LSN to GDN connection apparent. This loss of neurotransmitter release or production may also explain the gradual decay of the chemical connections over a three day period. However, the formation of a connection from the GDN to LSN after four days in culture would tend to suggest that this was not the case (see figure 19).

The synthesis of both serotonin and dopamine occur directly from amino acids. In the case of serotonin the amino acid is tryptophan and for dopamine tyrosine is the starting point. Both of these precursor amino acids were present in the L-15 medium used in the experiments. Any lesion in the biosynthesis of the neurotransmitters must therefore be due to the absence of, or non-function of, a protein in the neurones themselves. This could be a transport protein responsible for the uptake of the amino acids, an enzyme involved in the biosynthetic pathways or a protein involved in the recycling of released transmitter. Alternatively the biosynthetic pathway itself could be down-regulated.

Uptake of amino acids into molluscan neurones in cell culture has been previously demonstrated in *Aplysia* (Ambron *et al,* 1985), suggesting that this process is not effected by isolation of the neurone. The biosynthesis of the transmitters may be blocked by the lack of an enzyme. This could explain the initial presence of chemical connections, with transmitter being released from the neurones previous pool. However if the neurone was unable to replace this pool with newly

synthesised transmitter, then chemical transmission would stop. This seems an unlikely explanation for the elimination of chemical connections since continued release of neurotransmitter has been demonstrated from isolated neurones over several days .

In many cases no connections were recorded between GDN/LSN pairs. The lack of connections can be explained in terms of faults in the pre- and postsynaptic machinery, as for the B5 and B19 (see page 49). However, on the postsynaptic side the receptors on the GDN or LSN would not be linked directly to the appropriate ion channels, as is the case with the B19. In these cases the receptors, once activated by neurotransmitter, act via G-proteins which activate second messenger systems. These second messengers then cause the opening of the ion channel mediating the response. This means that faults in either the link from the receptor to the G-protein or the G-protein to the second messenger cascade, would prevent the expression of the postsynaptic response. A further point for a breakdown in synaptic communication in this system could be the second messenger cascade involved in bringing about the opening of the ion channel. The synthesis of second messenger molecules themselves, or intermediates in the pathway may be blocked in the isolated neurones, causing a failure in the cascade.

4.3 NEURONES PLATED ON CONSECUTIVE DAYS.

Neurones plated on consecutive days were seen to form both chemical and electrical connections. It has been demonstrated in *Aplysia* that the amount of contact between neurones is important in determining whether a connection forms between them (Hawver and Schacher, 1993). The amount of contact between neurones in consecutive day experiments should have been increased over that between neurones under the previous culturing method. However the technique did not lead to an increase in the number of chemical connections, or to a decrease in the number of electrical connections.

The amount of contact between the neurones may have been increased, but the role of fasiculation of neurites cannot be overlooked. It is possible that although contact between the neurones is occuring the neurites may just be passing over, rather than growing along, one another. Workers have shown that growth cones extend along specific

pathways in nervous systems, often including neurites and axons of other neurones (for example, see Kawada, 1986). These interactions are dependant upon the product of extracellular matrix components and cell surface adhesion molecules. The required extracellular matrix products may be absent from the conditioned medium used in these experiments, possibly removed by filtration of this medium before use.

Varicosities produced along the length of the neurites have been proposed to be release sites for neurotransmitter in *Aplysia* sensory neurones (Bailey and Chen, 1983). It is therefore possible that there may be a great deal of contact between neurites of the two neurones without any varicosities contacting the neurites of the partner cell. In such a case chemical transmission may not be observed.

Unlike the chemical connections obtained during the same day experiments, the direction of the chemical connections was not biased towards one neurone. Three connections were recorded from LSN to GDN and two from GDN to LSN. The direction of the connections appeared uneffected by which neurone was placed in culture first. From the previous work on neurones plated on the same day, the results gathered for neurones plated on consecutive days appear strange. It may have been expected that plating the LSN out first would lead to all subsequent connections being from the LSN to GDN. This was not the case however, with LSN to GDN forming twice and GDN to LSN forming once. This may be due to the small number of pairs tested, and possibly with further experimentation the previous situation would have been mirrored.

When neurones were plated on the same day and allowed to extend overnight, it is possible that release sites and newly synthesised receptors could be quite clustered along the neurites that the neurones had extended. In the consecutive day experiments, the neurone plated on day one would have been extending neurites for two days by the time recordings were made on day three. During this period the receptors and release sites could have become more spatially segregated over the neurites. The LSN could lose its dominance since the dopamine receptors along the neurites of the LSN may become more accessible. This could mean that the GDN would have been able to form connections to the LSN more readily. The problem with this argument is

that increased spatial arrangement and availability of receptors should increase the likelihood of chemical connections forming in either direction. This is not supported by the results obtained, although the number of pairs tested was small compared to that of the same day experiments.

The spacing of release sites and receptors along the neurites over time could help explain why the chemical connections detected between neurones plated on the same day are gradually eliminated. The connections obtained could have been due to close apposition of the pre- and postsynaptic machinery, without these elements being organised into a functional synaptic junction. Without any synaptic organisation, the elements on either side of the junction may be subject to being moved further apart. This would mean that the already weak connection between the two areas would be further weakened, or completely abolished. If this process was occuring at numerous sites along the neurites, then the overall connection between the two neurones would be seen to gradually weaken.

4.4 CIRCUITS FORMED IN CELL CULTURE.

The circuit experiments attempted were very inconclusive. Only a small number of the circuits constructed in culture survived to allow recordings to be made. In three of these four cases 'full' circuits were obtained, where all three neurones were connected with both of their circuit partners. This would seem to advocate the rationale of using the consecutive days plating technique to increase the area of contact between the neurones. Out of twelve possible connections in the four circuits, eleven were successfully formed. These results suggest that the presence of three neurones tends to make the formation of connections more likely. In the consecutive day experiments thirteen pairs of neurones were tested, and on five occasions no connection was present. In the circuit experiments, twelve individual neurone pairs were tested and on only one occasion was no connection detected.

In each circuit one LSN and the GDN were plated on the same day. The individual connections between these neurones were seen to be chemical on three occasions, from the LSN to the GDN. One of these connections was reciprocal. On only one occasion was no connection

detected. If this is compared to the experiments where the neurones were plated on the same day, chemical connections between the circuit 'pairs' appear more probable. Only twelve chemical connections were detected out of forty pairs in the original same day experiments, compared with three chemical connections out of four in the pairs involved in the circuits. The presence of a third neurone may therefore be having an effect that aids in the formation of chemical junctions. However it is difficult to draw firm conclusions from the small number of results obtained.

Within these circuits, as with the same day experiments, the LSN appeared to be the dominant prejunctional neurone over the GDN. Six chemical connections were detected between the LSN and GDN and only one from the GDN to LSN. The junction between the GDN and LSN was one half of a reciprocal chemical connection, and was very weak. The connections from the LSNs plated on day one to GDNs were chemical on three occasions and electrical on one occasion. This is different from the situation in the consecutive day experiments where the connections were evenly split between LSN to GDN and vice versa. Indeed the results mirror those from the experiments where neurones were plated on the same day, where LSN was three times more likely than GDN to be prejunctional. The conflicting evidence from the consecutive day and circuit experiments is probably due to the small numbers of pairs recorded.

The circuits showed that it was possible for a GDN to receive chemical connections from two identical neurones at the same time.These connections formed regardless of whether the two LSNs were chemically or electrically coupled. The GDN may not recognise that both inputs onto it are from identical neurones, leading to the GDN maintaining an inappropriate connection. Another possibility is that one of the connections would have eliminated, leaving only one of the LSN to GDN connections present. This was not tested since none of the circuits recorded from on day three survived fully intact for any further recording. Elimination of this type may have occurred due to competition between the two LSNs for a limited number of appropriate receptor sites on the GDN neurites and axon. Competition between the neurones for a limited amount of some trophic factor released by the GDN to signal to prospective prejunctional neurones could also account

for elimination (Hawver and Schacher, 1993).

The patterns of activity recorded from the circuits were difficult to analyse in much detail. In the case of circuit one the three neurones were not fully interconnected, and therefore no overall activity was seen. Circuit four had extremely weak connections between the neurones, none of which were apparent when all three neurones were simultaneously active. Weak interactions were seen in circuits two and three (see figures 27 and 30). The pattern of activity from these circuits appeared uncoordinated, probably due to a combination of weak interneuronal connections and the intrinsic membrane properties of the neurones involved.

In circuit two the electrical connection between the two LSNs was not sufficiently strong to synchronise the activity of these two neurones. If this had been the case then the GDN may have been inhibited without the need to strongly depolarise one of the LSNs. When both LSNs were active it may be thought that the GDN would have been inhibited due to the dual inhibitory inputs from the LSNs. This was not the case however, probably due to the weak nature of the inhibitory connections which the GDN was receiving. The recovery of the GDN from LSN inhibition did not have any effect on either LSN as predicted by the connectivity of the circuit.

The pattern of activity recorded from circuit three also showed weak interactions that could be predicted from the interneuronal connectivity. Weak inhibition was observed from LSN two to LSN one, and the GDN inhibited LSN two, but only when the GDN was strongly depolarised. LSN two and the GDN were connected by reciprocal inhibitory chemical connections. Neurones connected in this way have previously been shown to behave as predicted by mathematical models (Kleinfeld *et al*, 1990 b). In this model mutually inhibitory neurones fire alternate bursts of action potentials giving rise to a circuit with two stable output states. Although the reciprocal inhibitory connection between the GDN and LSN two in circuit three gives them the 'hard-wired' theoretical capability to form such a model, the connections and intrinsic properties of the neurones prevent it. The connections between the two neurones were weak, particularly from the GDN to LSN two. This meant that only strong depolarisation of the GDN would cause even a decrease in firing

frequency in the LSN. For its part LSN two was only able to inhibit the GDN when it was stimulated to give a high frequency burst of action potentials.

The half-centre model could have been shown if both neurones were at resting potential. At this point a depolarising current injection would be made to one of the neurones. If the connections between the neurones were strong enough, this would give rise to a hyperpolarisation of the second neurone. For the model to operate correctly with two neurones, the inhibited neurone should rebound strongly from the inhibition leading to this neurone firing action potentials. This activity in the second neurone would then inhibit the first neurone, which should then rebound from the inhibition to inhibit the second neurone. The two neurones should theoretically be able to continue with the cyclic activity without the need for further current injections. This situation did not arise with the GDN and LSN since these two neurones did not possess the capacity for postinhibitory rebound. The lack of this property means that the cyclical activity could only have been produced by injecting current into the neurones at the appropriate times (see Kleinfeld *et al,* 1990 b), or by having a third neurone providing phasic excitatory input to both neurones.

Rhythmic behaviour could occur without the need for repeated current injection if both neurones display endogenous bursting properties and are mutually inhibitory. If only one neurone was an endogenous burster then the second neurone would have to capable of rebounding from the inhibition it was receiving from the burster. Should the non-burster be capable of firing action potentials due to postinhibitory rebound, then the production of patterned output would be dependant upon the strengths of the connections. Neither the GDN or LSN were endogenous bursting neurones or were able to rebound from inhibition. This means that for any half-centre activity to be recorded between reciprocally connected GDN and LSN the activity would have to be artificially imposed by current injection.

As mentioned above the GDN and LSN did not show any postinhibitory rebound ability, which made the production of patterned output more difficult. In several cases (see figures 16 and 18) the two neurones can be seen to be inhibited from firing action potentials, and

then recover from the inhibition to continue firing. This return to an active state can be taken as a form of rebound, but the situation appears to be that the membrane potential of the inhibited neurone is merely returning to its previous level. The reason the neurone returns to its active state is probably due to the potential to which the neurone returns being greater than the threshold potential for the production of action potentials. Figure 17(a) shows an example where a non-active neurone was hyperpolarised. If the neurone was capable of postinhibitory rebound it may be expected that as it repolarises it would depolarise sufficiently to fire action potentials. The GDN and LSN therefore do not appear to exhibit the membrane property of postinhibitory rebound, as shown by neurones in the feeding pattern generator of *Lymnaea stagnalis* (Benjamin and Rose, 1979) and the swimming generator of *Clione* (Satterlie, 1985). It has been demonstrated in many circuits that output can be radically altered due to the effects of various neurotransmitters (see Getting, 1989; Harris-Warwick and Marder, 1991). Such experiments were not carried out in this present study, principally because the connections between the neurones were weak. Although individual neurones may have been effected by the applied substances, the effects on the overall circuit activity would have been difficult to gauge.

4.5 THE USE OF NEURONE CULTURE.

As mentioned in the introduction, neurone culture has become a much used technique in neurobiology both in invertebrate and vertebrate studies. Here the technique was used in an attempt to construct a small circuit of neurones and study the interactions between them. Although such circuits were eventually constructed, the experimentation on them was limited by the weak connections between the neurones, and the small number of circuits available. The latter of these was due to the number of neurones that could be successfully isolated from the ganglia and would survive in the culture conditions long enough for electrical recordings to be made. This was a large problem throughout the work, and although the neurones generally survived well once isolated, a great many neurones were lost during the initial isolation from the ganglia. On many occasions neurones were placed into culture, and despite appearing healthy, would not stick to the substrate or once they had, would not grow neurites. The isolation of neurones for cell culture can therefore be time-consuming and

technically problematic.

The appearance of chemical connections between neurones in culture has been shown in many different invertebrates (e.g. Fuchs *et al,* 1981; Camardo *et al,* 1983; Haydon, 1988; Syed *et al,* 1990) and vertebrates (e.g. Kraszewski and Grantyn, 1992). In this study the appearance of chemical connections appears an event that is down to luck. The connections appear to occur randomly, with some pairs which were obviously in physical contact giving rise to them (e.g. figure 14) whilst others which seemed in contact did not (e.g. figure 15). This apparent discrepancy is difficult to explain. Studies on synaptogenesis in cell culture would appear a time-consuming and frustrating enterprise from the results obtained in this study, mainly due to the unreliablity of chemical connections to form. However, other studies have shown that the culture technique is a viable way in which to investigate aspects of neuronal synaptogenesis.

The study of neuronal circuits in culture is open to the same problems as for the study of chemical connections between two neurones. Circuits have been reconstructed in culture conditions (Syed *et al,* 1990; Kleinfeld *et al,* 1990 b), but from the results obtained in this study neurone culture techniques would appear not best suited to the study of circuits. Considering the vast amount of knowledge that has been obtained by *in situ* experiments on such things as the crustacean stomatogastric ganglion, the *Tritonia* swimming system and the *Lymnaea stagnalis* feeding system, the need to use the culture technique at all in such studies is doubtful. The difficulty of reconstructing chemical connections and the time involved in the whole technique would appear to place more constraints on the experimenter than advantages. Future refinements to the culture methodology may lead to a situation where the technique becomes more reliable with regards to the formation of connections. This would leave the culture technique with great advantages for investigating aspects of circuits which are not easily assessible in whole ganglia preparations.

One interesting prospect for the use of cell culture techniques for studying connections and circuits has recently been reported (Sharp *et al,* 1992 and 1993). Here the neurones from the crab stomatagastric ganglion were artificially coupled by an electronic circuit. This method, although

having some technical limitations, would appear to offer investigators greater control over the neurones in culture, and does not rely on extension of neurites or the formation of connections. The method may therefore remove the element of luck that seems to be required for synapse formation between neurones in cell culture.

The technique of neurone culture has advantages over *in situ* recording for studying synapses, particularly where the formation of a synapse is to be followed. From the results presented in this study it can be seen that there are technical difficulties which need to be addressed before this useful technique can be fully applied to the investigation of synapses and circuits in cell culture. At present the neuronal cell culture technique appears more suitable for studies on neurite extension, growth cone behaviour and physiology and individual neurone pharmacology. For these types of studies the *in vitro* technique has a major advantage over *in situ* in that the axon, neurites and growth cones of the neurones are all easily accessible in an easily manipulated environment.

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